MODULATION OF TUMOR ANGIOGENESIS THROUGH THE USE OF ANTISENSE OLIGODEOXYNUCLEOTIDES TARGETED TO VEGF AND BFGF

By

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Dedicated to
my parents, Xuehui and Ying
my wife, Weiwen
and
my daughter, Julia.

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Abstract of Dissertation Presented to the Graduate School
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MODULATION OF TUMOR ANGIOGENESIS THROUGH THE USE OF ANTISENSE OLIGODEOXYNUCLEOTIDES TARGETED TO VEGF AND BFGF

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Angiogenesis is critical for the growth and metastatic spread of solid tumors. It is tightly controlled by specific regulatory factors. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been implicated as the key factors in tumor angiogenesis. The present studies were undertaken to evaluate the effects of blocking VEGF/bFGF production by antisense phosphorothioate oligodeoxynucleotides (AS-ODNs) on the angiogenic activity and growth of a preclinical model of renal cell carcinoma (Caki-1).

Efficient deliveries of AS-ODNs were achieved using cationic liposome (DOTAP:DOPE) based delivery systems both *in vitro* and *in vivo*.

AS-ODNs sequences against VEGF and bFGF have been designed and their efficacies were tested *in vitro*. Effective AS-ODNs against VEGF (V515) and bFGF (B460) were identified. Treatment of Caki-1 cells with V515 or B460 led to a reduction in VEGF or bFGF expression levels sufficient to impair the proliferation and migration

potential of co-cultured endothelial cells. The observed effects were AS-ODNs sequence specific, dose dependent and were achieved at a low, non-toxic dose. The treatment of Caki-1 cells with V515 or B460 was also sufficient to impair the Caki-1 tumor cell induced angiogenesis *in vivo*. When V515 or B460 treated Caki-1 cells were injected into nude mice and evaluated for their angiogenic potential, the number of vessels initiated were significantly reduced.

To test antitumor efficacy of VEGF/bFGF AS-ODNs treatment, V515 and B460 were administrated to Caki-1 xenograft tumor bearing mice. The results showed that systemic administration of VEGF/bFGF AS-ODNs significantly inhibited the growth of Caki-1 tumors. More importantly, a better response was observed when these two AS-ODNs treatments were combined. A combination of VEGF/bFGF AS-ODNs treatment with VEGF/bFGF receptor inhibitor or single dose local radiation also showed enhanced tumor responses when compared to single treatment alone.

These results indicate that AS-ODNs against pro-angiogenic factors VEGF and bFGF may have great utilities in the treatment of renal cell carcinoma either alone or in combination with other anti-cancer therapies.

CHAPTER 1
INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. In spite of ever increasing efforts to understand its process and improve treatment, its incidence in the population is rising. In the US, about 1 in 2 men and 1 in 3 women will develop cancer in their lifetime (American cancer society, 2002). As a cause of mortality overall in the Western World, cancer is second only to cardiovascular disease. Cancer can be treated by surgical removal or destroyed with toxic chemicals or radiation. However, these approaches all have drawbacks. Surgery will work for many primary tumors, but metastases are difficult to identify let alone remove at an early stage. Radiation and chemotherapy are generally toxic to normal cells as well. If even a few cancerous cells remain, they can proliferate to produce a resurgence of the disease; moreover, unlike the normal cells, cancer cells are genetically dynamic and may evolve resistance to the chemicals used against them. New therapeutic approaches providing more tumor-specific targeting still need to be exploited. One promising new development of cancer therapy is the anti-angiogenic strategies, following the recognition that tumor growth and metastasis depend on establishment of new blood vasculature (Folkman, 1971; Folkman, 1972b).

Tumor Angiogenesis

Angiogenesis is the formation of new blood vessels out of pre-existing capillaries. It is a sequence of events that is of key importance in a broad array of physiologic and pathologic processes (Folkman and Shing, 1992). While it plays a key role in

development, in adults, it is a rare event under normal physiological circumstances, occurring almost exclusively in the female reproductive system. Under normal conditions such as wound healing, the angiogenic process switches on and then off at the appropriate times indicating tight regulation of stimulatory and inhibitory factors (Hanahan and Folkman, 1996). However, angiogenesis can be activated with a variety of pathological conditions and occur in a less controlled manner (O'Reilly, 1997). These including cardiovascular diseases (athcrosclerosis), rheumatoid arthritis, diabetic retinopathy, psoriasis, etc (Folkman, 2001). In addition, angiogenesis is critical in the growth and metastatic dissemination of cancer (Folkman, 1995; Folkman, 1992; Folkman, 1972a; Folkman and Shing, 1992).

It has been observed for more than one hundred years that tumors appear to be more vascular than normal tissues. It was not until in the early 1970s that Drs Folkman and Denekamp put forward the idea that tumors are highly vascularized and thereby vulnerable at the level of their blood supply. This is the initial recognition of angiogenesis being a therapeutically interesting process in the area of oncology. Folkman proposed the hypothesis that angiogenesis was a requirement for the growth and metastatic spread of solid tumors (Figure 1-1) (Folkman, 1971; Folkman, 1972a). He further hypothesized that solid tumors could only grow to a size of ~1-2 mm in diameter without developing new blood supply, and if the development of vascular supply could be prevented, tumor growth could be limited to a small size (Folkman, 1971; Folkman, 1972a). This hypothesis implied that by destroying the newly developing vessels of the tumor, all the tumor cells supported by these vessels could also be killed (Denekamp J, 1972).

The process of angiogenesis consists of multiple, sequential, and interdependent steps (Figure 1-2). It begins with local degradation of the basement membrane surrounding capillaries, which is followed by the invasion of the surrounding stroma by underlying endothelial cells in the direction of the angiogenic stimulus. Endothelial cell migration is accompanied by the proliferation of endothelial cells and their organization into three-dimensional structures that join with other similar structures to form a network of new blood vessels (Figure 1-2) (Auerbach and Auerbach, 1994).

The process of angiogenesis is mediated by the balance between pro-angiogenic and anti-angiogenic factors. Angiogenesis is rapidly initiated in response to hypoxic or ischemic conditions. In all types of angiogenesis, under either physiologic or pathologic conditions, endothelial cell activation seems to be the first process to take place. In tumors, angiogenesis begins by mutual stimulation between tumor cells and endothelial cells by paracrine mechanisms (Gasparini, 1999). Cytokines from various sources including tumor and stromal cells are released in response to hypoxia or ischemia. It is suggested that vascular endothelial growth factor (VEGF) is a major player in angiogenesis initiation (Ziche et al., 1997). Besides affecting vasodilation and vascular permeability, VEGF can induce the expression of proteases and receptors important in cellular invasion and tissue remodeling and is able to prevent endothelial cell apoptosis (Ferrara and Keyt, 1997; Gupta et al., 1999). Following the releasing of pro-angiogeneic factors, endothelial cells can release proteolytic enzymes (matrix metalloproteinases, MMPs) to degrade the extracellular matrix for migration, proliferation, and endothelial penetration into new areas of the body (Stetler-Stevenson, 1999).

Endothelial cell proliferation and migration is stimulated by pro-angiogenic growth factors, like VEGF and bFGF. VEGF and bFGF are direct acting pro-angiogenic growth factors. BFGF exists in both low molecular weight form and high molecular weight forms due to alternative translation (Florkiewicz et al., 1991). It is suggested that during angiogenesis, low molecular weight bFGF binding to endothelial cell surface FGF receptors leads to increased motility, proliferation and proteinase activity, whereas the high molecular weight forms may act on endothelial cell proliferation after nuclear translocation (Gleizes et al., 1995; Klein et al., 1997). VEGF, besides its effect on angiogenesis initiation, also affects endothelial cell proliferation through high affinity receptors (KDR/flk-1 and Flt-1) expressed on endothelial cells (Ferrara, 1999; Veikkola and Alitalo, 1999). Finally, the neovasculature become mature and stable through the interaction of endothelial cells with extracellular matrix and mesenchymal cells. After endothelial cell proliferation and migration and maturation and formation of endothelial tube structures, surrounding vessel layers composed of mural cells need to be recruited. Endothelial cells may accomplish this *via* the synthesis and secretion of platelet-derived growth factor (PDGF), a mitogen and chemo-attractant for a variety of mesenchymal cells (George, 2001; LaRochelle et al., 2002). Subsequent differentiation of the mural precursor cells into pericytes and smooth muscle cells is believed to be cell-cell contact dependent process (Griffioen and Molema, 2000).

Anti-angiogenic Targets in the Treatment of Cancer

Understanding angiogenesis and its unique characteristics in tumor growth and metastasis has provided insights to a variety of ways to interrupt the process. During the past decade, research on anti-angiogenic agents has exploded and with ever increasing interest in its potential (Kerbel, 2000). We now have a much clearer understanding of

tumor angiogenesis, including key regulatory factors, differences between normal and tumor vasculature, along with endogenous inhibitors and methods to study and quantify angiogenesis (Kerbel, 2000).

The complex process of tumor angiogenesis of tumor provides multiple potential targets for anti-angiogenic strategies. The formation of new blood vessels involves basement membrane degradation, endothelial cell migration, endothelial proliferation and tube formation. Anti-angiogenic strategies under evaluation target at least one of the several stages (Figure 1-2). These strategies vary from regulation of angiogenic factor expression in tumors, to endogenous inhibitors of angiogenesis. Currently, there are over 80 clinical trials employing such strategies underway (http://cancertrials.nci.nih.gov/) reflecting the high pace of development. Based on the biological activities, these strategies can be categorized into several broad classes. The first class consists of MMP inhibitors, compounds that block the degradation of the basement membrane. The second class of agents includes those designed to inhibit endothelial cell function, such as TNP-470, thalidomide, endostatin, etc. The third class of agents specifically targets angiogenic growth factors. It includes trysine kinase inhibitors of VEGF/bFGF, antibodies or AS-ODNs against pro-angiogenic growth factors or their receptors. The last class of agents target survival factors of neovascular blood supply, such as intergrin antagonists, or anti-VEGF therapy (Reinmuth et al., 2001; Fidler et al., 2000).

I will now review some of the important angiogenic factors that have potential as therapeutic targets in anti-angiogenic therapies.

VEGF-A and Its Receptors

VEGF-A and its receptor system is among the most substantial mediators of angiogenesis. Considerable evidence has accumulated indicating that VEGF is an angiogenic cytokine of central importance. Its angiogenic activities have been demonstrated in numerous experimental models (Takeshita et al., 1994; Wilting et al., 1992; Wilting et al., 1993; Potgens et al., 1995; Kondo et al., 1995). The central role of VEGF in tumor angiogenesis has also been suggested. Over-expression of VEGF has been reported to occur in the vast majority of clinically important cancers (Zhu and Witte, 1999; Hemmerlein et al., 2001; Ferrara and Keyt, 1997). High serum and urine levels of VEGF have been associated with poor survival and treatment outcome to patients of different cancers (Hemmerlein et al., 2001; Maeda et al., 1996; Gasparini et al., 1997; Edgren et al., 1999; Sliutz et al., 1995). Tumor associated endothelial cells frequently demonstrate increased expression of VEGF receptors (Zhu and Witte, 1999). Moreover, high VEGF expression is found to associate with increased microvessel density and increased metastatsis in cancers (Zhu and Witte, 1999; Tsuji et al., 2002; Fontanini et al., 2002; Ng et al., 2001).

Disruption of VEGF signal transduction provides a potential effective target for anti-angiogenic approaches. Different strategies have been designed and evaluated. Specific VEGF antibodies are a way of stopping the angiogenic effects of this growth factor. Systemic administration of monoclonal VEGF antibody into tumor bearing nude mice significantly suppressed tumor growth in several tumor models (Kim et al., 1993; Borgstrom et al., 1998). A humanized antibody, rhuMAb-VEGF also has been developed and is undergoing Phase II clinical trials (Lin et al., 1999; Presta et al., 1997).

Alternatively, AS-ODNs or antisense RNA were also been used to directly disrupt VEGF protein expression and lead to inhibition of tumor growth in different tumor systems (Smyth et al., 1997; Ellis et al., 1996; Nguyen et al., 1998). Another approach is the coupling of a toxin to VEGF itself. When active parts of diphtheria toxin (DT390) are linked to $VEGF_{165}$ or $VEGF_{121}$, the chimeric molecule exerts highly selective toxic effects on endothelial cells. It disrupts neovascularisation in the chicken chorioallantoic membrane assay and slows down the growth of tumors in preclinical tumor models (Arora et al., 1999).

Blocking the interaction of VEGF with it receptor and receptor signal transduction pathway provides another option for anti-angiogenic treatment. VEGF receptors (flt-1, flk-1 and flt-4) are almost exclusively expressed on endothelial cells, with VEGFR-2 (flk-1) believed to play a central role in VEGF signal transduction (Ortega et al., 1999; Bematchez et al., 1999). Antibodies against VEGF receptors also showed efficacy in inhibiting tumor growth in preclinical tumor models (Brekken et al., 2000; Klement et al., 2000; Witte et al., 1998). Targeting VEGF receptor expression through the use of antisense also has been shown to be effective at inhibiting tumor angiogenesis and growth (Kamiyama et al., 2002; Marchand et al., 2002). Purified soluble VEGFR-1 binds VEGF with high affinity and blocks VEGF induced endothelial cell proliferation (Kendall et al., 1996). Over-expression of VEGF soluble receptor through gene therapy significantly inhibits tumor growth and metastasis and leads to higher survival rates (Goldman et al., 1998; Hasumi et al., 2002; Shiose et al., 2000; Takayama et al., 2000). Recently, small molecular compounds that can inhibit VEGF receptor tyrosine kinase activities have been developed and initial preclinical studies

showed promising anti-angiogenic and antitumor effects (Solorzano et al., 2001; Ning et al., 2002; Hess et al., 2001). To interfere in the binding of VEGF with its receptors, novel peptides have been developed (Fairbrother et al., 1998). This approach provides another potentially effective means to disrupt the VEGF signal transduction pathway and a provide treatment for cancer.

FGFs and Their Receptors

Fibroblast growth factor was originally identified as an activity in pituitary extracts that stimulates the proliferation of Balb/c 3T3 cells (Armelin, 1973; Gospodarowicz, 1974; Gospodarowicz, 1975). Currently, FGFs consist of a family of over 20 structurally related proteins (Basilico and Moscatelli, 1992;Ornitz and Itoh, 2001). They bind and activate high-affinity tyrosine kinases, FGFR 1-4 (Lee et al., 1989; Dionne et al., 1990; Ruta et al., 1989; Reid et al., 1990). Among all the FGFs, FGF-2 also called basic fibroblast growth factor (bFGF) is one most extensively investigated and important in angiogenesis.

BFGF was originally purified from bovine pituitary gland (Esch et al., 1985). It acts in a paracrine and autocrine manner and is released by tumor cells, macrophages or the extracellular matrix. BFGF can stimulate endothelial proliferation and also is chemotactic for endothelial cell migration (Gospodarowicz et al., 1987; Moscatelli et al., 1986; Moscatelli et al., 1986). It can also up-regulate other important pro-angiogenic factors like VEGF or plasminogen activator (Seghezzi et al., 1998; Montesano et al., 1986). Blocking bFGF expression and function can be achieved by using vaccine, antisense against bFGF or its receptors (Plum et al., 2000; Maret et al., 1995; Wang and

Becker, 1997; Redekop and Naus, 1995; Ensoli et al., 1994; Murphy et al., 1992; Becker et al., 1989).

Blocking the intrinsic tyrosine kinase activity of FGF receptors is a promising new approach in anti-angiogenic strategies targeting the bFGF signal transduction pathway. Some experimental compounds have been found to specifically block signaling of FGFR-1 and inhibit angiogenesis (Mohammadi et al., 1998; Perollet et al., 1998).

The Tie-angiopoietin System

The tie-receptor family consists of two know endothelial tyrosine kinases: TIE1 and TIE2/Tek. They are identified in vascular endothelium and hematopoietic cells (Dumont et al., 1992; Iwama et al., 1993; Partanen et al., 1992; Schnurch and Risau, 1993). Mice lacking TIE 1 or TIE 2 are lethal (Puri et al., 1995). Ties may represent the earliest endothelial cell lineage marker and may regulate the endothelial cell proliferation, differentiation, and proper patteming during vasculogenesis.

The first Tie-2 ligand, Aniopoietin-1 (Ang-1) was identified from human neuroepithelioma and mouse myoblast cell lines (Davis et al., 1996). Ang-1 is a novel endothelial regulatory factor that has been found to promote angiogenic remodeling by vascular supporting as well as vessel maturation and stabilization. Another related ligand, Ang-2 also has been found. However, binding of Ang-2 to Tie-2 did not induce phosphorylation of Tie-2 in endothelial cells. Moreover, it seems to block Ang-1 activity and suggesting Ang-2 may be antagonize the activation of Tie-2 (Maisonpierre et al., 1997). These findings together underline the feasibility to use the Tie-angiopoietin system to control angiogenesis (Lin et al., 1998). But before these molecules can be used for therapy, their effects on adult human vasculature and their interaction with other

angiogenic molecules during physiological and pathological angiogenesis need to be further investigated.

Angiogenin

Angiogenin is a 14 kD single chain basic protein found in human adenocarcinoma cells. It is a potent inducer of angiogenesis *in vivo*, which functions in the picomolar range (Vallee and Riordan, 1997; Fett et al., 1985). It stimulates the proliferation of endothelial cells and promotes the adhesion of endothelial and tumor cells (Soncin et al., 1994; Hu et al., 1997). Blocking angiogenin with a monoclonal antibody can impair subcutaneous tumor growth of a colon adenocarcinoma in a dose dependent manner (Olson et al., 1994). In 40-50% of the cases, growth of human breast carcinoma xenografts in athymic mice could be completely inhibited by a humanized version of the monoclonal antibody (Piccoli et al., 1998). In addition to its efficacy of inhibit tumor growth, it may also inhibited the establishment and metastatic growth of tumor cells (Olson et al., 2002). Besides the use of antibodies, other strategies to abolish angiogenin-induced angiogenesis include the use of DNA aptamers or antisense (Olson et al., 2001; Nobile et al., 1998). Anti-angiogenic therapy using angiogenin as a target may become an important tool because angiogenin mediates angiogenesis by mechanisms distinct from VEGF and bFGF (Lixin et al., 2001; Moroianu and Riordan, 1994).

Endogenous Inhibitors (Endostatin, Angiostatin)

Angiostatin and endostatin are two endogenous peptides that have been found to have potent anti-angiogenic effect (O'Reilly et al., 1997; O'Reilly et al., 1994b; O'Reilly et al., 1994a). Angiostatin is a prolytic fragment of plasminogen and endostatin is a 20 kD fragment of collagen XVIII. These factors make endothelial cells resistant to

angiogenic stimuli and induce "dormancy" of metastases. Administration of the recombinant protein, or expressing angiostatin or endostatin by means of gene therapy, illicits potent anti-tumor effects in the preclinical studies (O'Reilly et al., 1997; O'Reilly et al., 1994b; O'Reilly et al., 1994a; Bertolini et al., 2000; Jin et al., 2001; Yamanaka et al., 2001; Feldman et al., 2001; Sacco et al., 2001; Szary and Szala, 2001; Wen et al., 2001; Shi et al., 2002). The anti-tumor activities of angiostatin and endostatin are currently undergoing clinical evaluation.

In addition to angiostatin and endostatin, there are other endogenous angiogenic inhibitors including restin, vasostatin, etc (Pike et al., 1998; Ramchandran et al., 1999). Also, human prolactin, growth hormone, placental lactogen and growth hormone variant are angiogenic factors, whereas their 16 kD N-terminal fragments are anti-angiogenic (Struman et al., 1999).

Integrins

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits with large ectodomains and short cytoplasmic tails. They control cell motility, differentiation and proliferation via interactions with extracellular matrix molecules. Integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ are up-regulated on proliferating endothelial cells in angiogenic blood vessels during wound healing as well as in tumor vasculature (Brooks et al., 1994; Friedlander et al., 1996). The $\alpha_V\beta_3$ integrin, an adhesion receptor for extracellular matrix components with an exposed RGD sequence, is an attractive target for anti-angiogenic therapy. This integrin is almost exclusively present on the cell surface of activated endothelial cells, but absent on quiescent endothelium or other cell types (Eliceiri and Cheresh, 1999). Antibodies against $\alpha_V\beta_3$ were found to inhibit adhesion-dependent signal

transduction by angiogenic factors, leading to apoptosis of activated endothelial cells. Consequently, these compounds could block endothelial tube formation and angiogenesis in tumors (Brooks et al., 1994; Brooks et al., 1995). Currently, integrin antagonists are being evaluated in phase I and phase II clinical trials (Brower, 1999).

Matrix Metalloproteinases (MMPs) and Tissue Inhibitor of Metalloproteinases (TIMPs)

To form new blood vessels, endothelial cells of existing blood vessels must degrade the underlying basement membrane and invade into the stroma of the neighboring tissue (Mignatti and Rifkin, 1993; Mignatti and Rifkin, 1996). These processes of endothelial cell invasion and migration require the cooperative activity of the plasminogen activator and the MMPs.

The MMPs are a family of structurally related zinc-dependent endopeptideases collectively capable of degrading extracellular matrix (ECM). MMPs play an important role in the degradation of ECM, both in physiological conditions, such as morphogenesis and tissue repair and in pathologic conditions, such as tumor invasion and metastasis. The activity of MMPs is controlled at different levels (Liekens et al., 2001). First, the expression of MMPs is up-regulated by angiogenic growth factors (Giuliani et al., 1999; Bond et al., 1998; Wang and Keiser, 1998). Secondly, MMPs need to be activated proteolytically (Murphy et al., 1999). Lastly, the MMPs activities are also regulated by their inhibitors TIMPs (Blavier et al., 1999; Henriet et al., 1999). However, a large body of evidence suggests that this regulation is lost during tumor growth and metastasis (Rasmussen and McCann, 1997). An imbalance between MMPs and their TIMPs is responsible for the invasive phenotype of breast, colon and lung tumors and a low

survival rate in urothelial cancers (Kossakowska et al., 1996; Gohji et al., 1996b; Gohji et al., 1996a).

Inhibition of MMPs activities thus has been extensively studied as an approach to inhibit growth and invasion of neoplastic cells. Important anigiogenesis inhibitors in clinical trials based on MMP blocking are Metastat, Neovastat, BMS-2752291, Mariamstat, AG3340, Bay 12-9556 and CGS 27023A (Vihinen and Kahari, 2002). MMPs inhibitors currently in clinical trials are synthetic peptides or non-peptidic molecules, chemically modified tetracyclines, bisphosphonates or natural MMP inhibitors (neovastat). Further trials using MMPs in combination with classical chemotherapy are also underway.

Plasminogen Activator (uPA) and its Inhibitors (PAI-1)

Proteases of the fibrinolytic cascade also contribute to the regulation of angiogenesis. Expression of urokinase-type plasminogen activator (uPA) by malignant cells results in an aggressive phenotype with increased tumor angiogenesis and metastatic invasion. PAI-1, the natural uPA inhibitor, is paradoxically also up-regulated in human tumor samples (Landau et al., 1994). Clinically, expression of both, uPA and PAI-1 correlate with a poor prognosis of several cancers (Rosenquist et al., 1993; Heiss et al., 2002; Osmak et al., 2001). Taken together, inhibition of uPA rather than PAI-1 activity might be a possible therapeutic target to treat cancer and other angiogenesis dependent diseases.

Thrombospondin (TSP)

Extracellular matrix molecules play an important role in maintaining tissue integrity and endothelial cell viability. However, thrombospondin, one such extracellular

matrix molecules, first identified in 1979 from platelets is also a very powerful inhibitor of endothelial cell adhesion, migration, motility and proliferation and angiogenesis *in vivo* (Lawler et al., 1978; Taraboletti et al., 1990; Good et al., 1990). TSP-1 is a member of a family of structurally related proteins encoded by different genes, which includes 4 recent identified members, TSP 2-5 (Bornstein and Sage, 1994). TSP-1 inhibits endothelial cell proliferation, migration and can induce endothelial apoptosis (Vogel et al., 1993; Tolsma et al., 1993). In addition, tumor cells transfected with TSP1 developed smaller tumors than the parental cell lines (Volpert et al., 1998; Streit et al., 1999). In clinical studies, expression of TSP1 has been inversely correlated with malignant progression of breast cancer, melanoma, and lung carcinomas (Zabrenetzky et al., 1994). These data indicate that TSP-1 can be utilized to inhibit tumor growth by an anti-angiogenic mechanism.

Platelet Factor 4 (PF-4)

Platelet factor 4 belongs to the CXC cytokine superfamily (Strieter et al., 1995). It is a 7.8 kD protein of 70-amino acid in length that shares homologies with β-thromboglobulin and interleukin-8 (Deuel et al., 1977). It has been known for a while that PF-4 inhibits angiogenesis (Maione et al., 1990). First, PF-4 inhibits endothelial cell proliferation, migration and angiogenesis *in vivo* (Gupta and Singh, 1994; Maione et al., 1990). Second, PF-4 is targeted to endothelial cells that undergo angiogenesis *in vivo* (Hansell et al., 1995), Moreover, it has been shown the tumor angiogenesis could be inhibited by PF-4 (Sharpe et al., 1990; Kolber et al., 1995). In addition, human glioma cells infected with a secretable PF-4 cDNA grew slowly *in vivo* and were hypovascular

(Tanaka et al., 1997). Finally, data exist suggesting the PF-4 may counteract angiogenic factor activity at the sties of platelet activation (Watson et al., 1994).

Administration of recombinant PF-4 protein or delivery of the PF-4 gene showed efficacy against tumor growth in preclinical models (Maione et al., 1990; Tanaka et al., 1997; Kolber et al., 1995; Maione et al., 1991). These findings suggest that it may have great potential as an effective anti-angiogenic factor in the treatment of cancer. Currently, recombinant PF-4 is being evaluated in clinical trials (Belman et al., 1996).

Interleukins

Interleukins have been known for a long time for their immunomodulatory activities but their role in angiogenesis is just becoming a hot topic in cancer research. Some of the interleukins have anti-angiogenic properties (interleukin-10, -12, -18) while others seems to be pro-angiogenic (interleukin-1, -6, -8, -15), some may even have both effects (interleukin-4). The mechanisms by which interleukins achieve their effects on endothelial cells are quite different and not fully understood (El Awad et al., 2000; Huang et al., 1996; Voest et al., 1995). Interleukins may be useful tools to treat angiogenesis-related diseases including cancer. However, better understanding of their specific functions, as well as their interactions is needed.

Anti-angiogenic Factors - Summary

Among all these angiogenic regulatory factors, the most important pro-angiogenic growth factors in cancer are VEGF and bFGF. Both factors are found to stimulate endothelial cell proliferation and migration (Leung et al., 1989; Plate et al., 1992; Schweigerer et al., 1987). Indeed, the expression of VEGF has been related to fundamental features of tumors, such as growth rate (Kim et al., 1993; Nagao and

Nishikawa, 1989), microvessel density (Toi et al., 1994; Straume and Akslen, 2002) and vascular architecture (Drake and Little, 1999; Faridi et al., 2002) as well as the development of tumor metastasis (Weidner et al., 1991; Faridi et al., 2002). A correlation between VEGF and/or bFGF expression and survival has been noted in some cancer patients (Gasparini et al., 1997; Yiangou et al., 1997; Dietz et al., 2000). Given the importance of VEGF and bFGF in the angiogenic process of cancer, these two growth factors were chosen as the targets for the presents investigations.

Antisense Oligodeoxynucleotides Technology

In order to inhibit the expression or function of specific gene products, such as VEGF and bFGF, a strategy with promise is the use of antisense oligodeoxynucleotides (AS-ODNs).

In 1977, it is first described that gene expression can be modified with exogenous nucleic acids by using single strand DNA to inhibit translation of a complementary RNA in a cell-free system (Paterson et al., 1977). Soon after, Zamecnik and Stephenson also demonstrated that AS-ODNs targeted to 3' end of virus could inhibit viral replication *in vitro* (Zamecnik and Stephenson, 1978). These initial findings showed that AS-ODNs could inhibit gene expression in a sequence specific manner. One of the first studies showing *in vivo* activities of AS-ODNs was published in 1991 (Whitesell et al., 1991). Since then, particularly with the introduction of efficient methods for DNA sequencing and ODNs synthesis, various targets have been analyzed *in vitro* and in animals with encouraging results (Jansen et al., 1998; Tamm et al., 2001; Pawlak et al., 2000; Golden et al., 2002; Braasch and Corey, 2002; Corey, 2002).

The essential steps in drug design are the identification of an appropriate target responsible for a certain disease and the development of a drug with specific recognition of and affinity to that target. For most conventional drugs the mechanism of fairly broad. In contrast, the basis of the use of AS-ODNs is that the introduction of ODNs complementary to target mRNA sequences into the cytoplasm can result in decreased expression of the gene being targeted (Dias N and Stein, 2002). Since AS-ODNs inhibit gene expression in a sequence dependent way, selective alteration of specific gene expression is possible. The AS-ONDs approaches generally used are mainly to inhibit oncogene expression, to induce apoptosis, to overcome multidrug resistance, or to inhibit pro-angiogenic growth factors (Pawlak et al., 2000).

In 1998, the first antisense drug (fomivirsen) was approved by the US Food and Drugs Administration (FDA) for the treatment of cytomegalovirus-induced retinitis in AIDS patients (de Smet et al., 1999). Although fomivirsen is administrated locally, the approval shows the feasibility of AS-ODNs as drugs for the treatment of human diseases.

With continuous development and understanding of tumor biology, inappropriate expression of certain genes was found to be basic to the pathophysiology of cancer. Consequently the use AS-ODNs as therapeutic strategies in the treatment of cancer has attracted much attention and intensive investigation. The currently more than 8 ongoing clinical trials illustrate the growing interest in AS-ODNs in the treatment of cancer (Koller et al., 2000; Tamm et al., 2001). Besides these approaches, AS-ONDs may also have a role in overcoming multidrug resistance in cancer. For example, the use of anti-*mdr*1 AS-ODNs has been shown to lead to reduction of the gene product gp170

expression, restore chemotherapy drug sensitivity, and even lead to eventual prolongation of survival (Cucco and Calabretta, 1996; Kuss et al., 2002; Pan et al., 2001).

Oncogene over-expression is one of the most common molecular events that may lead to cancer development. AS-ODNs are specific tools to inhibit expression of certain oncogenes and so can be used as potential drugs to reverse the harmful effects of dysregulated gene expression. AS-ODNs against *ras, myc, myb, bcr-abl*, as well as viral oncogenes, such as E6, E7 or HBx have been evaluated (Gray et al., 1993; Szczylik et al., 1996; Venturelli et al., 1990; Leonetti et al., 1996; Citro et al., 1994; Szczylik et al., 1991; Beer-Romero et al., 1997; Lappalainen et al., 1996). Methods to regulate the mechanisms of cell death and survival in order to shift the balance toward apoptosis are of great interest in the treatment of cancer. Studies have focused on targeting the vital anti-apoptotic genes, such as bcl-2, p53 and CRIPTO and MDM-2 proteins using AS-ODNs (Ziegler et al., 1997; Campbell et al., 1998; Normanno et al., 1999; Chen et al., 1998).

The application of AS-ODNs to target the pro-angiogenic growth factors is a new and promising strategy in cancer management. Studies with AS-ODNs against VEGF showed such treatment can significantly impair tumor angiogenesis and lead to tumor growth inhibition in VEGF dependent tumors (Masood et al., 2001; Masood et al., 1997). AS-ODNs directed at inhibiting the expression of bFGF also showed both anti-angiogenic and anti-tumor efficacy (Wang and Becker, 1997). The strategy of using AS-ODNs against both VEGF and bFGF as therapeutic intervention of cancer was explored in detail in studies described in this dissertation.

To date proof of clinical efficacy of AS-ODNs in oncology is very limited (de Smet et al., 1999). However, data providing proof of principle exist. Future development of AS-ODNs holds considerable promise in the treatment of cancer. Further development of new various new targets, assessment of combination treatments with several PS-ODNs, and investigations focused on strategies targeting tumor mechanisms should improve their therapeutic activities.

Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the sixth leading cause of cancer death in the United States, accounting for 3% of adult malignancies. There were an estimated 30,800 RCC cases diagnosed in 2001, with approximately 12,100 deaths in the United States (Jemal et al., 2002). The incidence of RCC deaths in the United States has been steadily increasing during the past 25 years, possibly partly because of increased sensitivity and greater use of various imaging modalities (Chow et al., 1999; Homma et al., 1995; Jayson and Sanders, 1998).

Although the etiology of RCC is unknown, several risk factors, including obesity, smoking, hypertension, diuretic use, consumption of fired meat, asbestos exposure, petroleum exposure, and frequent analgesic use have been consistently implicated (Dhote et al., 2000). Renal transplantation, with its associated immunosuppression, acquired cystic kidney disease also increase the risks of developing RCC (Hoshida et al., 1999). RCCs are clinically, histologically, and genetically a very heterogeneous group of tumors. Clear cell RCC is the most common type of RCC, accounting for over 70% of the cases. It is a highly vascularised neoplasm demonstrating clear evidence of abundant angiogenesis and abnormal blood vessel development (Yoshino et al., 2000).

Clinically, RCC patients can present with a multiplicity of manifestations ranging from the classic presenting triad of hematuria, pain and palpable renal mass to more obscure symptoms, such as those of paraneoplastic syndromes. Unfortunately, the classic triad usually indicates patients with far advanced disease, and it is seen in less than 10% of patients at presentation (Gibbons et al., 1976). More commonly, renal tumors are discovered incidentally during the course of various diagnostic studies.

The treatment of choice for RCC is surgical removal. Radical nephrectomy is accomplished by early ligation of the renal artery, renal vein, and *en bloc* removal of the kidney with the surrounding Gerota's fascia (Robson et al., 1969). Despite the remarkable improvements and response rates of single and combination chemotherapy in some solid tumors, RCC remains a chemoresistant tumor. The most common agents, vinblastine and floxiuridine, have response rates of 7% and 16%, respectively (Yagoda et al., 1995). In a review of 72 agents, evaluated in 3500 patients, between 1983 and 1992, an overall objective response rate of only 5.6% was found, mostly of short duration (Yagoda et al., 1995). Hormone therapy has been found to be equally ineffective (deKernion and Lindner, 1982). Consequently, there currently is no role for chemotherapy or hormone therapy in the treatment of RCC. Unlike chemotherapy, radiotherapy has been shown to provide some benefits to patients with RCC (Rost and Brosig, 1977). Preoperative radiation can reduce the risk of tumor dissemination at the time of nephrectomy; reduce primary tumor size; increase resectability and reduce tumor vascularity. Postoperative radiation therapy has the theoretical benefit of providing local control of tumor in patients with positive surgical margins, incompletely resected primary tumors, or lymph node involvement (Riches, 1966; Mantyla et al., 1977). Still, currently,

radiation therapy in RCC is generally reserved for palliation, most often for symptomatic bony metastases (Halperin and Harisiadis, 1983).

Taken together, five-year survival rates after radical nephrectomy for stage I RCC is approximately 94%, and stage II 79%. Patients with renal vein or inferior vena caval involvement have a survival rate of 25-50%, and patients with regional lymph node involvement or expracapsular extension have a survival rate of 12-25%. Five year survival rate for patients with stage IV disease is less than 5%.

The unsatisfactory management of RCC with conventional anticancer therapies warrants novel approaches to augment the tumor response and treatment outcome. Histopathological studies of RCC reveal it to be a highly vascularised neoplasm demonstrating clear evidence of abundant angiogenesis and abnormal blood vessel development (Figure 1-3) (Yoshino et al., 2000). Therefore it may provide an excellent target for anti-angiogenic therapeutic approaches. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are of particular interest. Both factors have been shown to be expressed in renal cell carcinoma tissues and renal cell carcinoma cell lines (Mydlo et al., 1993; Gospodarowicz et al., 1986; Mydlo et al., 1988; Sato et al., 1999; McLaughlin and Lipworth, 2000; Ferrara and Keyt, 1997). Serum levels of VEGF and bFGF often are elevated in RCC patients (Nguyen et al., 1994b; Fujimoto et al., 1991; Wechsel et al., 1999; Tomisawa et al., 1999; Paradis et al., 2000) and renal cell carcinoma VEGF and bFGF mRNA levels have been reported to be much higher than those found in surrounding normal tissues (Tricarico et al., 1999; Thelen et al., 1999; Tsuchiya et al., 2001; Eguchi et al., 1992). In addition, elevated serum/urine bFGF levels have been shown to associated with malignant progression and poor treatment outcome

(Song et al., 2001; Jacobsen et al., 2000; Rasmuson et al., 2001; Edgren et al., 1999; Dosquet et al., 1997; Fujimoto et al., 1995; Plunkett and Hailey, 1990; Miyake et al., 1996; Nguyen et al., 1994a; Huang et al., 1996). Taken together, these findings suggest that VEGF and bFGF are key factors involved in the angiogenic process of RCC. For these reasons, RCC was the tumor of choice for the current investigation of anti-angiogenic therapeutic approaches with the use of VEGF and bFGF AS-ODNs. An RCC cell line (Caki-1) was used. This cell line is a human clear cell renal cell carcinoma originally derived from a 49 year-old Caucasian male patient (Fogh, 1978). It grows *in vitro* as an anchored cell culture and also forms tumor in athymic nude mice. The histology of the Caki-1 xenograft displays many of the features of clinical samples of RCC (Figure 1-5).

Significance

Angiogenesis is unique process that contributes to a variety of pathologic processes and is especially critical to tumor growth and metastasis. It therefore has been proposed and utilized as both a prognostic indicator as well as a possible target for therapeutic intervention in certain malignant states. In the present studies, the feasibility of inhibiting tumor angiogenesis by utilizing AS-ODNs directed against VEGF and bFGF were investigated. A human renal cell carcinoma cell line grown *in vitro* or as solid tumor xenografts in nude mice was used as the tumor model. Through the investigations, a viable means of intervening with the angiogenic process and in situ growth of renal carcinoma cells was developed. It is further believed that this approach will not be confined to RCC, but will be applicable to other neoplasms and may even provide a basis for selective intervention in other diseases characterized by angiogenesis.

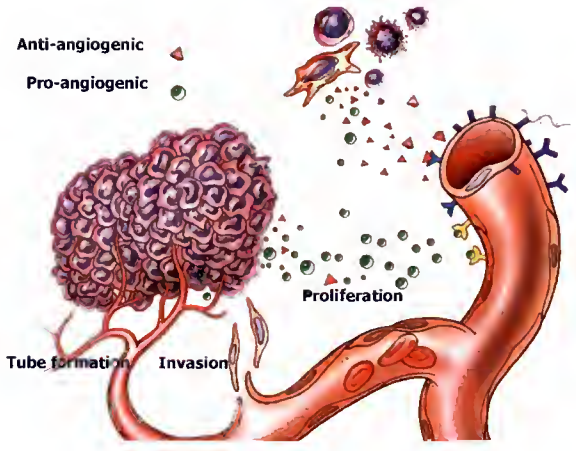


Figure 1-1. The angiogenic process. Tumor cells or host cells secrete pro-angiogenic growth factors, which then bind to specific receptors on endothelial cells. This ligand-receptor interaction leads to endothelial cell proliferation, migration, invasion and, eventually, capillary tube formation (Fidler et al., 2000).

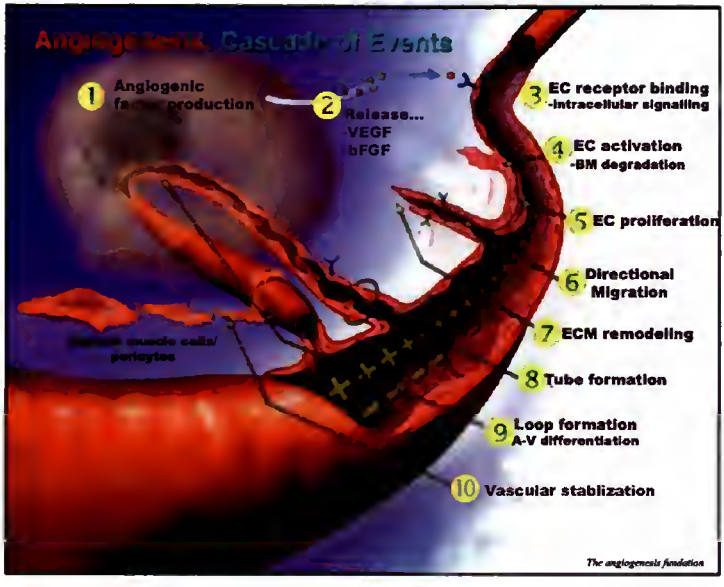


Figure 1-2. Cascade of events in tumor angiogenesis. (Source: The Angiogenesis Foundation, www.angio.org)

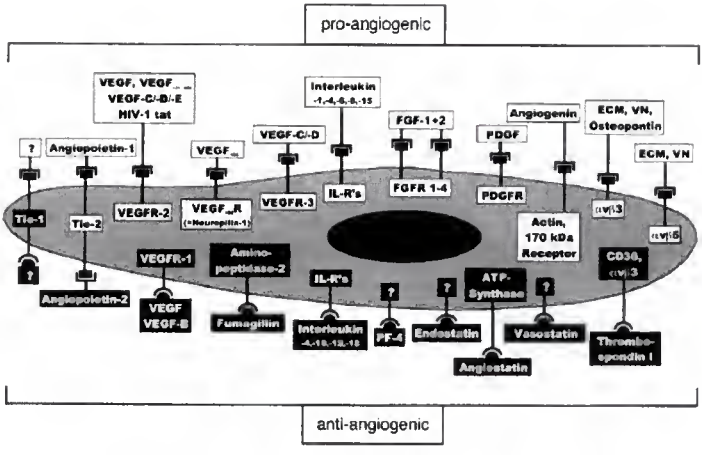


Figure 1-3. Major regulators of angiogenesis and their receptors. On the upper part of the cell pro-angiogenesis regulators are shown, on the lower part are inhibitory molecules. (Hagedorn and Bikfalvi, 2000)

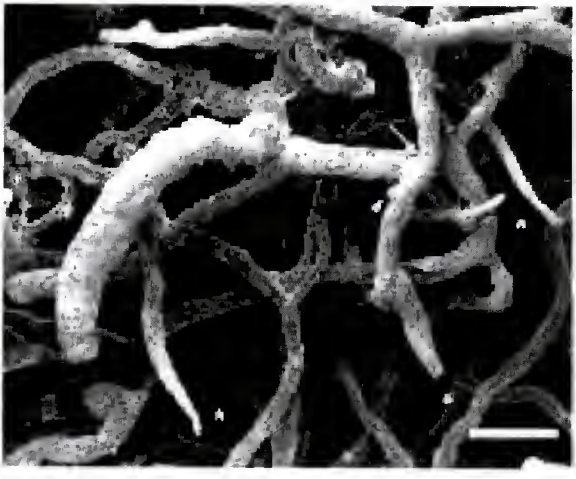


Figure 1-4. Resin cast of RCC tumor microvascular network. (Gerwins et al., 2000)

Figure 1-5. H&E staining histology section of Caki-1 xenograft tumor.

CHAPTER 2
CELLULAR DELIVERY OF ANTISENSE OLIGONUCLEOTIDES

Introduction

Many of the limitations of current cytotoxic therapies of cancer result from a lack of specificity of the anti-cancer agents. The advances in molecular biology over the past The application of two decades have made possible the concept of genetically based, targeted treatment. Antisense oligonucleotides (AS-ODNs) is one approach to specifically inhibit gene expression (Stein and Cheng, 1993; Wagner, 1994). These molecules, usually 18-20 bases in length, can undergo Watson-Crick hybridization to target mRNAs, ultimately resulting in decreased expression of the gene products. AS-ODNs technology has attracted great interest and shown great promise as agents to inhibit the expression of specific genes that regulate physiological functions or mediate various diseases (Harrison, 1993; Wagner, 1994; Wagner, 1995; Pan et al., 2002; Mani et al., 2002; Morris et al., 2002).

However, one of the biggest challenges in the application of AS-ODNs as therapeutic agents is the development of ways to maximize their cellular uptake (Wagner, 1995; Crooke, 1993). AS-ODNs are negatively charged molecules that behave as polyanions. In general this property leads to poor cellular uptake and intracellular distribution. Furthermore, the commonly used phosphorothioate modified AS-ODNs have quite high affinity for proteins, especially heparin-binding proteins (Fennewald and Rando, 1995). In addition, some new cell surface ODNs-binding proteins have also been identified recently (Beltinger et al., 1995; Hawley and Gibson, 1996). Even after

internalization into the cell through endocytosis (Yakubov et al., 1989; Crooke et al., 1995), naked AS-ODNs are localized to endosomes or lysosomes, that are topologically still "outside" of the cell (Tonkinson et al., 1994). ODNs may then either be released from the cell *via* exocytosis or may be partially digested (Tonkinson et al., 1994). Still, exists a growing literatures of antisense effects after naked AS-ODNs delivery (Anfossi et al., 1989; Gewirtz and Calabretta, 1988). Nontheless, it should be recognized that these effects were achieved at high AS-ODNs concentration and may be the result of AS-ODNs release into the cytoplasm through spontaneous endosomal/lysosomal rupture.

Many techniques have thus been then used to enhance AS-ODNs uptake, the most widely used being based on the application of cationic lipids. Cellular uptake as well as the activity in cell cultures can be improved greatly by cationic liposomes (Bennett et al., 1992; Lappalainen et al., 1994; Zelphati and Szoka, Jr., 1996b; Zelphati and Szoka, Jr., 1996a). Highly polar, water soluble molecules including AS-ODNs can be entrapped in the internal aqueous space of the liposme, while the lipids form into bilayers. Cationic liposomes spontaneously bind the negatively charged AS-ODNs and protect them against degradation. The macromolecular complexes have a positive charge at the surface, this results in a high affinity for most cell membranes, which are negatively charged under physiological conditions. Following the attachment to the membrane, the complexes are taken up *via* endocytosis. To help facilitate the release from endosomes and lysosomes, a helper lipid such as 1,2-dioleoyl-3-sn-phosphatidylethanolamine (DOPE) is often used in the liposome preparation. This inverted-cone-shaped lipids thought to facilitate cytosolic release through the fusion and disruption of endosomal membranes (Farhood et al., 1992). The flip-flop of anionic phospholipids in the endosome membrane, lead to

neutralization of the cationic lipid charge, displacement of the bound oligonucleotides, and release form the endosome (Figure 2-1) (Koltover et al., 1998; Lebedeva et al., 2000). Studies have demonstrated that ODNs can readily dissociate from the liposome complexes and are in bioavailable form within the cells (Tari, 2000; Abe et al., 1998).Cationic liposomes facilitated delivery of AS-ODNs has proven to be effective in many different cell lines and to be of general utility (Bennett et al., 1992).

However, studies in mammalian cell lines have demonstrated that AS-ODNs efficacy varies with different cationic lipids and lipid complexes, cationic lipid/DNA ratio, and cell type (Flanagan and Wagner, 1997; Lappalainen et al., 1997). This suggests that as a general rule, the best liposome composition and optimal liposome/ODNs ratio needs to be established for each cell lines to achieve best results.

In the present studies, fluorescein isothioicyanate (FITC) labeled ODNs were used to study the cellular uptake of ODNs using a cationic liposome delivery system (Noonberg et al., 1992). Although the biological activity of AS-ODNs against the molecular target is highly sequence-dependent, this is typically not the case for pharmacokinetics and toxicology. Indeed, the pharmacokinetics and toxicology of AS-ODNs of widely differing sequences directed against vastly disparate gene products have proven surprising similar (Srinivasan and Iversen, 1995). Thus, the same ODNs sequence was used for the present cellular uptake and toxicity studies.

Materials and Methods

Cell Culture

The clear cell RCC cell lines Caki-1, Caki-2 and A498 were gifts from Dr. Susan Knox (Stanford University). These cells were grown in Dulbecco's modified minimum essential medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal

bovine serum (FBS, Invitrogen, Grand Island, NY), 1% penicillin-streptomycin (Invitrogen, Grand Island, NY) and 1% 200 mmol/L L-glutamine (Invitrogen, Grand Island, NY).

FITC Labeled Phosphorothioate Oligodeoxynucleotides

The 20-mer ODNs, sequence: 5'- CAC CCT GCT CAC CGC ATG GC -3' (20-mers) were customer synthesized by Geno Mechanix (Alachua, FL). The entire backbone was phosphorothioate modified and FITC was labeled at the 5' end of the ODNs. The ODNs were suspended in sterile and endotoxin-free water at a concentration of 1 mM, aliquoted and stored at -20°C.

Liposome Preparations

Cationic liposomes of different lipid compositions were obtained from Dr. Jeffrey Hughes' lab (University of Florida, Gainesville, FL). DOTAP:DOPE is composed of cationic lipid 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) and a helper lipid 1,2-dioleoyl-3-sn-phosphatidylethanolamine (DOPE) at a molar ration of 1:1. DS3DOPC is composed of 1,2-Dioleoyl-sn-Glycero-3-Phophoserine-N-Citraconyl and dioleoyl-phosphatidylcholine (DOPC) at a molar ratio of 1:1. DOGSDSO is composed of 1',2'-dioleoyl-sn-glycero-3'-succinyl-2-hydroxyethyl disulfide ornithine conjugate (Tang and Hughes, 1998). CHDTAEA is composed of cholesteryl hemidithiodiglycolyl tris(aminoethyl)amine (Tang and Hughes, 1999). PEG-PE is composed of DOTAP, DOPE and polyethylene glycol distearoyphospatidylethanolamine (PEG-PE) at a molar ration of 25:25:3 (Meyer et al., 1998). All the lipids were obtained from Avanti Polar-Lipids (Alabaster, Al).

Briefly, the lipid mixture was evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The resulting lipid film was dried by nitrogen for an additional 10 min to evaporate any residual chloroform. The lipid film was re-suspended in sterile water to a final concentration of 1 mg/ml based on the weight of cationic lipid. The resultant mixtures were shaken in a water bath at 35°C for 30 min. The suspensions then were sonicated using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) for 1 min at room temperature to form homogenized liposomes. The particle-size distribution of liposomes was measured using a NICOMP 380 ZLS instrument (Santa Barbara, CA). The average particle diameter was 144.0 ± 77.0 nm. Liposomes were stored at 4°C and used within 3 months.

Antisense Treatment

Caki-1, Caki-2, A498 cells were set at 1×10^5 in 60 mm dishes and allowed to attach overnight. For comparison of delivery efficiency by different liposomes, FITC labeled ODNs were mixed with different liposomes in serum free medium or 10% FBS medium and incubated at room temperature for 30 min. For other studies, only DOTAP:DOPE liposome was used. The medium of cells was then changed with that containing ODNs-liposome complex at a ODNs concentration of 1 μM/ml and incubated at 37°C for 3 hours. Equal amounts of 20% FBS medium were added to dishes and continued to incubate for a total of 24 hr, except for the time course studies, in which, cells were incubated for different lengths of time.

Fluorescence Microscope

After FITC labeled ODNs treatment, the medium containing ODNs was removed and cells were washed 4 times with PBS. The cells were then fixed in 1% p-

formaldehyde for 1 hr. Fluorescent microscope pictures were then taken using a Zeiss Axioplan 2 Florescence Microscope (Zeiss, Thornwood, NY) made available by the Optical Microscopy Facility at Brain Institute, University of Florida.

Flow Cytometry Analysis

After FITC labeled ODNs treatment, the medium containing AS-ODNs was removed and cells were washed 4 times with PBS. The cells were then collected by trypsin digestion. After fixation in 1% p-formaldehyde for 1 hr, cells were re-suspended in PBS at the concentration of 1×10^6 cells/ml and kept in the dark. Green fluorescent intensities of the cells derived from FITC were then analyzed by FACS on a Becton Dickinson flow cytometer made available through the University Cole Facility for Flow Cytometry at the University of Florida.

Toxicity Studies

Caki-1 cells were set in 96-well dishes at 1×10^4 cells per well and allowed to attach overnight. The culture medium was then changed to 100 µl serum free or 10% FBS medium containing various doses of DOTAP:DOPE. The cells were incubated for 24 hr at 37°C. The viable cells after treatment were measured using a CellTiter96AQueous Assay System (Promege, Madison, WI). Briefly, 100 µl of phenylmethasulfazone (PMS) solution was added to 2 ml 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tet razolium (MTS) solution and mixed by gentle swirl. 20 µl of the combined MTS/PMS solution was added into each well and incubated at 37°C for 2 hr. After incubation, 25 µl of 10% SDS was added into each well to stop the reaction. The absorbance was then measured at 490 nm using a microplate reader.

Results

Uptake of FITC labeled ODNs measured by flow cytometry and fluorescence microscopy, allowed the assessment of cellular delivery of ODNs. Delivery of naked ODNs resulted in poor internalization into the cells, either in serum free or 10% FBS conditions. Only a small portion of cells treated showed moderate increases in fluorescence intensity derived from FITC (Figure 2-2). However, the use of cationic liposomes (DOTAP:DOPE) was found to significantly improve the up-take efficiency of ODNs in Caki-1 cells, in both serum free and 10% FBS conditions (Figure 2-2).

In order to optimize the delivery system for ODNs, other cationic liposome compositions were evaluated for their delivery efficiency of ODNs in Caki-1 cells. The cells were treated with FITC labeled ODNs delivered by different cationic liposomes (DOTAP:DOPE, DS3DOPC, DOGSDSO, CHDTAEA and PEG-PE) for 24 hr at a AS-ODNs concentration of 1 μM/ml. Both serum free and 10% FBS conditions were studied and compared. Significant enhancements of ODNs cellular up-take were observed in all liposome treatment groups, with DOTAP:DOPE and DS3DOPC being most efficient and with minimal serum resistance (Figure 2-3). Since DOTAP:DOPE is of simple composition and easy to prepare, it was chosen as the delivery vehicle for the rest of studies.

Studies to optimize the delivery efficiency of ODNs by DOTAP:DOPE were then carried out. One major factor that determines the delivery efficiency is the liposome to ODNs charge ratio. In order to determine the optimal charge ratio in Caki-1 cells, the cells were treated for 24 hr with 1 μM/ml FITC labeled ODNs prepared with different amount of DOTAP:DOPE to achieve various charge ratios (1, 1.25, 1.5, 1.75, 2, 2.5). The

fluorescent intensities of Caki-1 cells after the treatment were then compared (Figure 2-4). Increases in delivery efficiency and as well as resistance to serum were observed as the charge ratio increased. A plateau in these effects occurred at a charge ratio of about 2. This charge ratio was used in all subsequent studies evaluating the delivery of AS-ODNs in Caki-1 cells.

The delivery efficiency of ODNs by DOTAP:DOPE as the function of time was then evaluated. Caki-1 cells were treated with 1 μM/ml FITC labeled ODNs delivered by DOTAP:DOPE at the pre-determined optimal charge ratio of 2. The fluorescent intensity of Caki-1 cells after different lengths of treatment were determined by FACS (Figure 2-5). Very fast up-take of ODNs by Caki-1 cells were observed when delivered by DOTAP:DOPE liposomes. The up-take of ODNs by Caki-1 cells reached a plateau within 24 hr of incubation (Figure 2-5).

With the optimized delivery system based on cationic liposomes (DOTAP:DOPE) very efficient cellular up-take of ODNs was achieved in Caki-1 cells. Significant up-take of FITC labeled ODNs was observed in Caki-1 cells after 24 hr treatment of 1 μM ODNs delivered by DOTAP:DOPE at a charge ratio of 2. ODNs were delivered into ~100% of the cells with uniform cellular distribution and enhanced nuclear concentration (Figure 2-6). This result was achieved in both exponential phase and plateau phase Caki-1 cells (Figure 2-7).

This optimized delivery system was then further tested for ODNs delivery efficiency in two other RCC cell lines. The results showed that similar highly efficient cellular up-take of ODNs also could be achieved in Caki-2 and A498 cells (Figure 2-8)

ODNs as fragments of DNA sequences have very low toxicity (Rubenstein et al., 1997; Agrawal et al., 1997). The toxicity of the cationic liposome delivery vehicle was also examined in both serum free and 10% FBS conditions. No significant toxicity of DOTAP:DOPE in Caki-1 cells was observed with doses up to 150 mg/ml in 10% serum and 100 mg/ml in serum free conditions. At doses higher than 100 mg/ml some cytotoxicity was observed using DOTAP:DOPE in serum medium. The DOTAP:DOPE dose used in the delivery studies was only 10 mg/ml, which resulted in no toxicity in Caki-1 cells either in serum free or 10% FBS conditions.

Discussion

AS-ODNs can block the expression of specific target genes involved in the development of human diseases. Therapeutic applications of antisense techniques are currently under investigation in many different fields. In order for AS-ODNs to down-regulate gene expression, it must penetrate into the target cells. Phospholipid bilayers represent a strong barrier to the movement of ions. Studies on the diffusion of ODNs through model membranes have led to the general conclusion that it is of little importance. Meanwhile, internalization of ODNs into cells has been clearly demonstrated, implying the existence of other mechanisms other than passive diffusion (Garcia-Chaumont et al., 2000). To date, the precise mechanisms involved in ODNs penetration are still not totally clear. Though it has been found that up-take of AS-ODNs can occur through receptor mediated active transport (Wu-Pong et al., 1994), which depends on temperature (Loke et al., 1989; Yakubov et al., 1989), the structure and the concentration of ODNs (Vlassov et al., 1994), and the cell lines. At the present time, it is generally believed that adsorptive endocytosis and fluid phase endocytosis are the major mechanisms of ODNs internalization (Dias N and Stein, 2002; Garcia-Chaumont et al.,

2000). At relatively low ODNs concentrations, it is likely that internalization occurs via interaction with a memberane-bound receptors (Loke et al., 1989; Yakubov et al., 1989; de Diesbach et al., 2000). While at higher ODNs concentrations, these receptors are saturated, and the endocytotic process assumes larger importance. Numerous reports have demonstrated that naked ODNs are internalized poorly by cells (Gray et al., 1997; Stein et al., 1993)(Figure 2-2). More importantly, naked ODNs tend to localize in endosomes/lysosomes, where they are unavailable for antisense purposes. As has been demonstrated in numerous experiments, the *sine qua non* of antisense activity appears to be nuclear localization (Dias N and Stein, 2002).

To improve cellular uptake and ODNs spatial and temporal activity, delivery vehicles were developed. Among them, cationic liposomes are most commonly used. Cellular up-take of ODNs, as well as their activity in cell cultures, can be improved by cationic liposomes (Bennett et al., 1992; Lappalainen et al., 1994; Zelphati and Szoka, Jr., 1996b; Zelphati and Szoka, Jr., 1996a). Cationic liposomes are safe, simple and easy to produce on a large scale (Nabel et al., 1993; Nabel et al., 1994a; Nabel et al., 1994b). They have been approved by FDA for clinical use. However, when tested for their ability to promote delivery of FITC-ODNs in mammalian cell lines, their efficacy varied significantly (Lewis et al., 1996). Important variables include cationic lipsome/ODNs ratio, composition of lipid and cell type tested (Flanagan and Wagner, 1997; Lappalainen et al., 1997; Vellon et al., 2002). This suggests that even though cationic liposomes have been proven to be effective and to be of general utility, a best reagent and optimal liposome/ODNs ratio could and should be empirically established for each cell type.

In the present studies, delivery efficiencies of FITC tagged ODNs by different liposome compositions were first evaluated. All cationic liposome compositions significantly enhanced cellular up-take of ODNs. However, the incorporation of PEG-PE significantly inhibited the delivery efficiency of cationic liposomes (DOTAP:DOPE). PEG-PE, when incorporated into conventional liposomes, can provide a steric barrier at the surface of lipsomes that inhibits opsonization, and therefore can extend the residence time of liposomes in the blood (Webb et al., 1998). However, even though the use of PEG-PE in liposomes have minimal effect on the binding and subsequent endocytosis of lipid/DNA complexes, it did severely inhibit the endosomal release of AS-ODNs into the cytoplasm (Figure 2-3) (Song et al., 2002).

Given the efficiency of delivery, minimum serum resistance and simplicity of liposome composition and preparation, DOTAP:DOPE was chosen as the delivery vehicle for AS-ODNs in Caki-1 cells (Figure 2-3). A major problem associated with cationic liposomes is low transfection efficiency due to inactivation of cationic liposomes by serum (Felgner et al., 1987). Much effort has been devoted to resolving this problem. The charge ratio of liposome to DNA has been proven previously to be critical for high efficiency of lipofection and serum resistance (Yang and Huang, 1997; Yang and Huang, 1998). The optimal transfection efficiency and minimal serum inactivation was achieved at a DOPTA:DOPE/ODNs charge ratio of about 2 (Figure 2-4). Using this charge ratio, cellular up-take of ODNs by Caki-1 cells reaches a plateau within 24 hr (Figure 2-5). ODNs in Caki-1 cells were evenly distributed in the cytoplasum with enhanced nuclear concentration (Figure 2-6). This distribution is believed to best facilitate antisense function of AS-ODNs (Wagner, 1994; Hogrefe, 1999). With the optimized delivery

system, efficient cellular up-take of ODNs were achieved in both plateau and exponential phases Caki-1 cells (Figure 2-7) as well as two other RCC cell lines (Caki-2, A498) (Figure 2-8).

Cytotoxic evaluation of DOTAP:DOPE confirmed that cationic liposomes are safe unless very high doses are used (Porteous et al., 1997; Gao and Huang, 1995). The dose used in the present studies (10 mg/ml) was far below the doses that resulted in cytotoxic effects in Caki-1 cells (>100 mg/ml)(Figure 2-9).

In conclusion, cationic liposomes (DOTAP:DOPE) enhanced the cellular up-take of ODNs in RCC cell lines. The simplicity of preparation, efficiency of up-take and safety features have rendered the cationic liposome (DOTAP:DOPE) an attractive vehicle for AS-ODNs therapy.

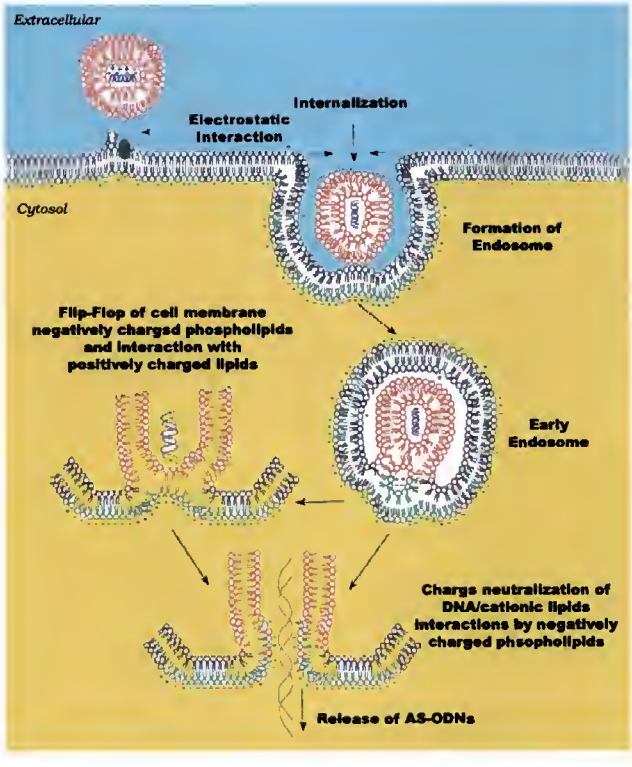


Figure 2-1. Proposed mechanisms of internalization of cationic liposome into cells and release of ODNs in cytoplasm. Modified from Lebedeva (Lebedeva et al., 2000).

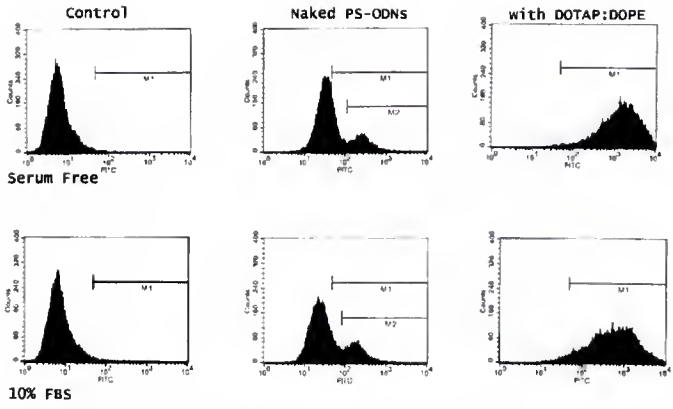


Figure 2-2. Flow cytometry histogram of Caki-1 cell fluorescece intensity after treatments of 1 μM FITC labeled ODNs either with or without DOTAP:DOPE liposome.

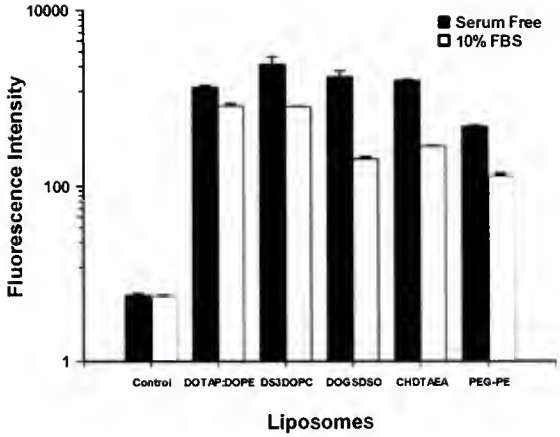


Figure 2-3. Efficiency of cellular up-take of FITC labeled ODNs delivered by different liposmes in Caki-1 cells. Caki-1 cells were treated with 1 μM FITC labeled ODNs in serum free or 10% FBS conditions for 24 hr. Each bar represents the mean + S.E. of 3 independent experiments.

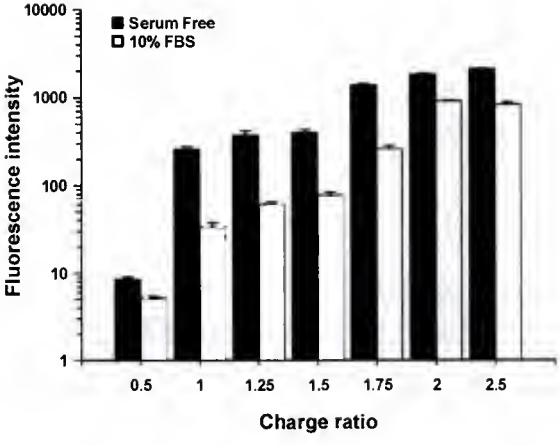


Figure 2-4. Effect of liposome to ODNs charge ratio on the delivery efficiency of ODNs in Caki-1 cells. Caki-1 cells were treated for 24 hours with 1 μM FITC labeled ODNs delivered by different amounts of DOTAP:DOPE liposomes at the charge ratio indicated. Each bar represents the mean ± S.E. of 3 independent experiments.

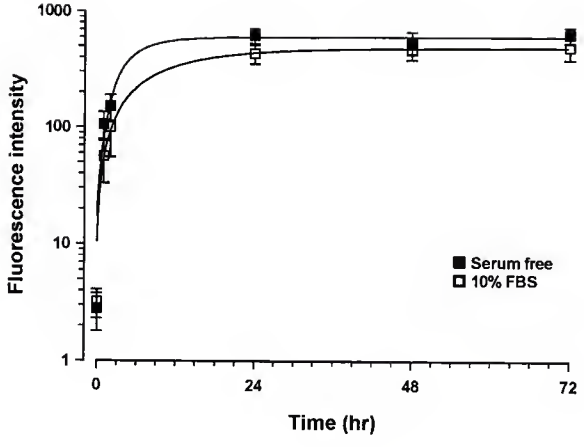


Figure 2-5. Time course of up-take of ODNs delivered by DOTAP:DOPE in Caki-1 cells. Caki-1 cells were treated with 1 μM FITC labeled ODNs delivered by DOTAP:DOPE liposome at a charge ratio of 2.0. Results are the mean ± S.E. of 3 independent experiments.

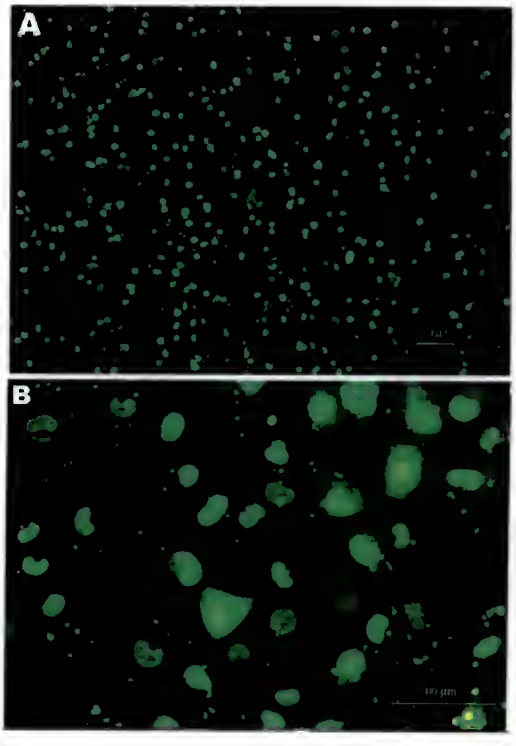


Figure 2-6. Fluorescent microscopic pictures of Caki-1 cells after FITC labeled ODNs treatment. Caki-1 cells were treated with FITC labeled ODNs for 24 hr at a dose of 1 μM. A) low magnification, 5x; B) high magnification, 20x.

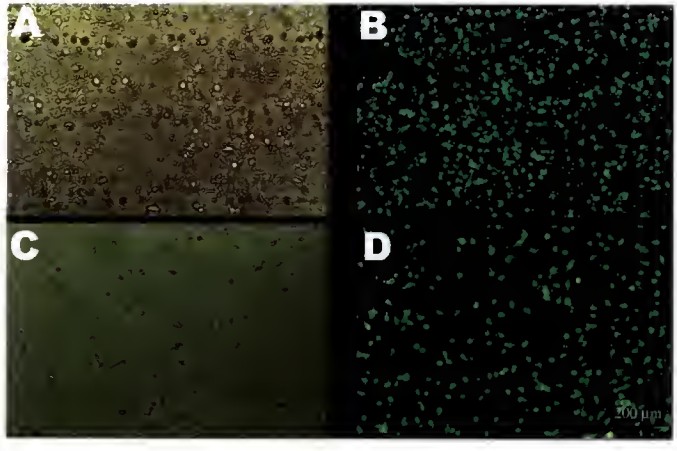


Figure 2-7. Fluorescent microscopic pictures showing cellular uptake of FITC labeled ODNs in Caki-1 cells. Caki-1 cells were treated with FITC labeled ODNs at a dose of 1 µM for 24 hr. A) bright field picture of confluent Caki-1 cells; B) fluorescent field picture of confluent Caki-1 cells; C) bright field picture of exponential phase Caki-1 cells; D) fluorescent field picture of exponential phase Caki-1 cells.

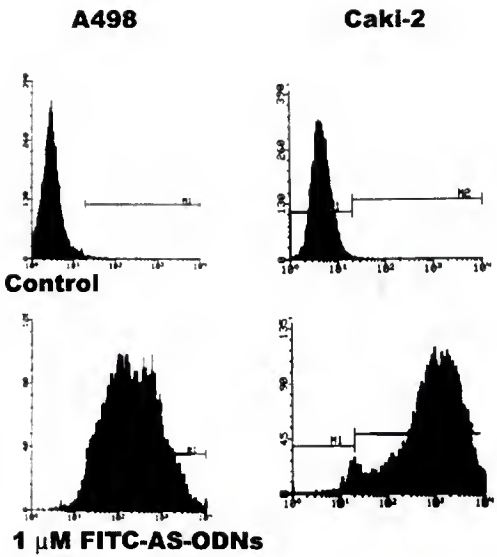


Figure 2-8. Delivery efficiency of FITC labeled ODNs by DOTAP:DOPE liposome in other RCC cell lines (A498, Caki-2).The cells were treated with FITC labeled ODNs at a dose of 1 μM for 24 hr.

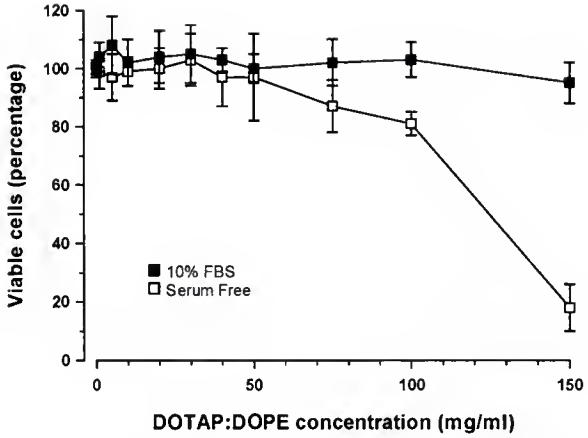


Figure 2-9. Caki-1 cell viability after 24 hr treatment with different concentrations of DOTAP:DOPE liposomes in serum free or 10% FBS conditions.

CHAPTER 3
AS-ODNS DESIGN AND *IN VITRO* ASSESSMENT

Introduction

Angiogenesis, a complex multi-step process involving the formation of new blood vessels from pre-existing ones, is tightly regulated by both positive and negative regulatory factors (Risau, 1997). These regulators, which include pro-angiogenic factors such as basic fibroblast growth factor (bFGF) (Montesano et al., 1986), angiogenin (Gho and Chae, 1997) and vascular endothelial growth factor (VEGF) (Leung et al., 1989; Saleh et al., 1996; Asano et al., 1995; Borgstrom et al., 1996; Cheng et al., 1996), as well as angiostatic peptides such as endostatin (O'Reilly et al., 1997; Perletti et al., 2000), angiostatin (O'Reilly et al., 1994a; O'Reilly et al., 1994b; O'reilly et al., 1994) and thrombospondin (Folkman and Shing, 1992; Folkman, 1995) are potential targets for anti-angiogenic therapy of solid tumors (Smith et al., 1999; Bicknell and Harris, 1992; Denekamp, 1999; Bicknell R and Harris A.L., 1992; Folkman, 1971; Denekamp J., 1999). Among all these factors, VEGF and bFGF are believed to be most important regulators in tumor angiogenesis (Risau, 1997;Siemeister et al., 1998).

VEGF is an endothelial cell specific mitogen, secreted as a 45 kDa homo dimer protein. There are five human isoforms derived from alternative splicing (VEGF 121, 145, 165, 189, 206) as illustrated in Figure 3-1 (Tischer et al., 1991; Houck et al., 1991; Poltorak et al., 1997). $VEGF_{121}$ and $VEGF_{165}$ are the only soluble isoforms and also the most abundant, with $VEGF_{165}$ being the major isoform and most powerful stimulator of endothelial cell proliferation (Houck et al., 1992; Soker et al., 1997). $VEGF_{165}$ is

commonly expressed in a wide variety of human and animal tumors (Hanahan and Folkman, 1996) and has been shown to induce angiogenesis both *in vitro* and *in vivo* (Leung et al., 1989; Plate et al., 1992a; Plate et al., 1992b). It is currently believed that this diffusible molecule is probably a key mediator of tumor angiogenesis (Ferrara, 1999a; Ferrara, 1999b). Indeed, the expression of VEGF has been related to fundamental features of tumors, such as growth rate (Kim et al., 1993), microvessel density (Toi et al., 1994) and vascular architecture (Drake and Little, 1999) as well as the development of tumor metastasis (Weidner et al., 1991). A correlation between VEGF expression and survival has been noted in some cancer patients (Gasparini et al., 1997; Maeda et al., 1996).

Basic fibroblast growth factor is a prototype of a large family of 13 structurally related, heparin-binding growth factors. It affects the growth, differentiation, migration and survival of a wide variety of cell types (Bikfalvi et al., 1997). BFGF was originally purified from the bovine pituitary gland as a 146-amino acid protein with a molecular weight of 15 kD (Gospodarowicz, 1975). It was later found to represent a proteolytic product of the primary 18 kD form (Bikfalvi et al., 1997). The amino acid sequence of 18 kD bFGF is highly conserved among species with 89-95% identity among human, bovine and rat (Abraham et al., 1986a; Abraham et al., 1986b). This low level of divergence suggests that there may be functional importance for all regions of bFGF. Larger forms of bFGF have also been identified resulting from alternative CUG translation starting sites (Florkiewicz et al., 1991a; Florkiewicz et al., 1991b). The use of different in-frame CUG codons upstream of the conventional AUG start codon allows translation of several bFGF isoforms with different molecular weight (Figure 3-6) (Okada-Ban et al., 2000). In

addition to the 18 kD isoform, alternative translation of 22, 22.5, 24 and 34 kD isoforms are also possible (Amaud et al., 1999). The main structural feature of the four high molecular weight forms of bFGF is the presence of nuclear localization sequence which directs the growth factors to the nucleus, whereas the 18 kD bFGF isoform initiated from AUG start codon is essentially cytosolic.

BFGF is a multifunctional growth factor which has various effects in a large panel of cells and tissues. It plays key role in development, remodeling and disease states in almost every organ system (Bikfalvi et al., 1997). One of best characterized activities of bFGF is its ability to regulate the growth and function of vascular cells such as endothelial cell and smooth muscle cells. BFGF also regulates the expression of several molecules thought to mediate critical steps during angiogenesis. These include interstitial collagenase, urokinase type plasminogen activator (uPA), plasminogen activator inhibitor (PAI-1), uPA receptor, and β1 integrins (Montesano et al., 1992; Mignatti and Rifkin, 1993; Klein et al., 1993). . It is a potent angiogenic factor involved in tumor angiogenesis and metastasis (Basilico and Moscatelli, 1992). Up-regulation of bFGF and its receptors have been found in tumor tissues compare to normal tissues (Smith et al., 1999; Dellacono et al., 1997; Arbeit et al., 1996). Clinically, associations between serum/urine bFGF and cancer outcome have been shown in several tumor systems, including RCC (Wechsel et al., 2000; Edgren et al., 1999; Nanus et al., 1993; Fujimoto et al., 1991), breast cancer (Yiangou et al., 1997), head and neck cancer (Dietz et al., 2000), cervical cancer (Sliutz et al., 1995), liver cancer (Poon et al., 2001), pancreas cancer (Ohta et al., 1995), thyroid cancer (Sasaki et al., 2001) and glioma, neuroblastoma (Bredel et al., 1997; Komuro et al., 2001).

In light of their important roles in tumor angiogenesis, VEGF and bFGF may be attractive targets for anti-angiogenic therapeutic interventions applied to the treatment of cancer. Attempts to abrogate the angiogenic activity of VEGF and bFGF have focused on inactivating VEGF/bFGF through the use of antibodies against VEGF/bFGF or their receptors (Mordenti et al., 1999; Kim et al., 1993; Aonuma et al., 1999; Lu et al., 2002; Brekken et al., 2000) and VEGF soluble receptors (Lin et al., 1998), inhibiting VEGF/bFGF receptor tyrosine kinases (Hennequin et al., 1999; Laird et al., 2002; Solorzano et al., 2001; Ning et al., 2002) or suppressing VEGF and bFGF messages (Smyth et al., 1997; Ellis et al., 1996a; Ellis et al., 1996b; Nguyen et al., 1998a; Nguyen et al., 1998b; Inoue et al., 2000). The latter relied on antisense oligonucleotides (AS-ODNs) or antisense RNA (Eguchi et al., 1991; Mercola and Cohen, 1995a; Mercola and Cohen, 1995b) to modulate gene expression by disrupting RNA expression. AS-ODNs technology provides an approach for inhibiting gene expression with target specificity as a particular advantage (Stein and Cheng, 1993; Engelhard, 1998a; Engelhard, 1998b). AS-ODNs are also easy to produce in large quantities which make them potentially more practical than antisense RNA vector delivery approaches.

In the present studies, AS-ODNs against VEGF and bFGF were designed and their efficacy tested *in vitro* in the model of human RCC (Caki-1).

Materials and Methods

Cell Culture

The clear cell RCC cell lines Caki-1, Caki-2 and A498 were gifts from Dr. Susan Knox (Stanford University, CA). Caki-1 cells were grown in Dulbecco's modified minimum essential medium (DMEM, Invitrogen, Grand Island, NY) supplemented with

10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY), 1% penicillin-streptomycin (Invitrogen, Grand Island, NY) and 1% 200 mmol/L L-glutamine (Invitrogen, Grand Island, NY).

Antisense Phosphorothioate Oligodeoxynucleotides (AS-ODNs)

Antisense and control ODNs (20-mers) were custom synthesized by Geno Mechanix (Alachua, FL). AS-ODNs V515 was complementary to 5' UTR just up-stream of the translation start site (AUG codon) of VEGF mRNA: 5' - CTC ACC CGT CCA TGA GCC CG - 3'. Scramble sequence: 5' – CAC CCT GCT CAC CGC ATG GC - 3'; sense sequence: 5' - CGG GCT CAT GGA CGG GTG AG - 3' and an inverted sequence: 5'-GCC CGA GTA CCT GCC CAC TC - 3', were used as controls ODNs. AS-ODNs B460 was complementary to the translation start site (AUG codon) of bFGF mRNA: 5' TCC CGG CTG CCA TGG TCC CT 3', AS-ODNs B471 was complimentary to the coding region of bFGF mRNA: 5' CGT GGT GAT GCT CCC GGC TG 3'; AS-ODNs B931 was complimentary to the 3' UTR: 5' GAT GTG GCC ATT AAA ATC AG 3'. Scramble sequence: 5' GCC TGG ACC CTG GCT CTC TC 3'; sense sequence: 5' AGG GAT GGC TGC CGG GA 3' and an inverted sequence: 5' TCC CTG GTA CCG TCG GCC CT 3' were used as controls. All AS-ODNs were suspended in sterile and endotoxin free water at a concentration of 1 mM, aliquoted and stored at -20°C.

DOTAP:DOPE Liposome Preparation

Cationic liposomes were prepared using the method described by Tang (Tang and Hughes, 1999). Briefly, cationic lipid 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) was dissolved in chloroform and mixed with a helper lipid 1,2-dioleoyl-3-sn-phosphatidylethanolamine (DOPE) (Avanti Polar-Lipids, Alabaster, Al) at a molar ratio

of 1:1. The mixture was evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The resulting lipid film was dried by nitrogen for an additional 10 min to evaporate any residual chloroform. The lipid film was re-suspended in sterile water to a final concentration of 1 mg/ml based on the weight of cationic lipid. The resultant mixtures were shaken in a water bath at 35°C for 30 min. The suspensions then were sonicated using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) for 1 min at room temperature to form homogenized liposomes. The particle-size distribution of liposomes was measured using a NICOMP 380 ZLS instrument (Santa Barbara, CA). The average particle diameter was 144.0 ± 77.0 nm. Liposomes were stored at 4°C and used within 3 months.

VEGF Enzyme Immunoassay

Caki-1 cells (1×10^5) were set in 60 mm dishes and allowed to attach ovemight. The medium then was removed and replaced with AS-ODNs in serum free medium with liposome (DOTAP:DOPE) and incubated for 5 hr. Fresh medium containing 10% FBS then was added. After 24 hr of incubation, or at different time points for the time course studies, the VEGF concentration was determined in the medium using a human VEGF ELISA kit (R &D Systems, Minneapolis, MN).

Enzyme Immunoassay of bFGF

Caki-1 cells (1×10^5) were set in 60 mm dishes and allowed to attach ovemight. The medium then was removed and replaced with AS-ODNs in serum free medium with liposome (DOTAP:DOPE) and incubate for 5 hr. Fresh medium containing 10% FBS then was added. Caki-1 cells were collected 72 hr later, washed and suspended 1×10^6 cells in 1 ml PBS containing protease inhibitors (100 μg/ml Phenylmethanesulphonyl

fluoride, 20 μg/ml leupeptin, 3 μg/ml aprotinin). The suspension was subjected to 3 freeze-thawing cycles, ultrasonication for 5 s (100 W) on ice, and was centrifuged at 14,000 g for 10 min. The supernatant containing the intracellular bFGF was used for the bFGF concentration determination (human bFGF immunoassay kit, R & D Systems, Minneapolis, MN).

VEGF and bFGF Relative Quantitative RT-PCR

Caki-1 cells were set at 3×10^5 in 100 mm dishes and allowed to attach overnight. The cells were then treated with 1 μM VEGF antisense (V515), bFGF antisense (B460) or control ODNs as described. 24 hr later the cells were collected and the total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) and RNA concentrations were determined by UV spectrophotometry. A 2 μg total RNA sample was used to reverse synthesize cDNA using Superscript II reverse transcriptase (Invtrogen, Grand Island, NY). A 2.5 μl aliquot of the reverse transcriptase reaction product then was used for the PCR reaction. VEGF PCR reactions were carried out with a VEGF gene specific relative RT-PCR Kit (Ambion, Austin, TX). BFGF PCR reactions were carried out using a forward primer: 5'GCA GCC GGG AGC ATC ACC A 3' and reverse primer: 5' GCC CAG TTC GTT TCG GTG CCC A 3' (Campbell et al., 1999). The PCR reactions were run 22 cycles (denature 94°C 30 s, anneal 60°C 60 s, extension 72°C 60 s) in a DNA Engine 200 (MJ research, Waltham, MA). PCR products then were run in 2% agrose gel and stained by ethidium bromide. The gels were visualized and analyzed (Gel Doc 2000 gel documentation system, Bio-Rad, Hercules, CA). All PCR preparations were carried out in a laminar flow hood using aerosol resistant plugged pipette tips.

FGF Receptors 1-4 RT-PCR

Total RNA of exponential phase Caki-1 cell was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) and RNA concentrations were determined by UV spectrophotometry. A 2.5 μl aliquot of the reverse transcriptase reaction product then was used for the PCR reaction. Primers for human FGFR 1-4 designed by Tartaglia etc were used (Tartaglia et al., 2001). The PCR reactions were run for 30 cycles (denature 94°C 30 s, anneal 60°C 60 s, extension 72°C 60s) in a DNA Engine 200 (MJ research, Waltham, MA). The specificity of the cDNA amplifications were then verified by endonuclease restriction analyses (Tartaglia et al., 2001). All PCR preparations were carried out in a laminar flow hood using aerosol resistant plugged pipette tips. Negative controls without template DNA were included in each assay. 18S primer set (Ambion, Austin, TX) was used as positive control.

Cell Cycle Assays

Caki-1 cells were plated in 60 mm dishes at 2×10^5 cells per dish and allowed to attach overnight. The cells were then treated with 1 μM B460 or control ODNs mixed with DOTAP:DOPE as described above. 48 hr later, the cells were trypsinized, counted and fixed in 50% ethanol overnight. Before analyzed by FACS, the cells were treated with 1 mg/ml RNase (in PBS) for 30 min. The samples were then washed with PBS twice and resuspended in 25 mg/ml propidium iodine (PI) in PBS at a concentration of 1×10^6 cells/ml. The cells were stained with PI in darkness for 15 min and were analyzed by FACS for cell cycle distribution on a Beckman Dickinson flow cytometer made available through the University of Florida Core Facility for Flow Cytometry.

Apoptosis Assays

Caki-1 cells were set in 2-well chamber slides and treated with 1 μM B460 or control ODNs as described earlier. 48 hr later, the cells were washed and fixed in 4% para-formaldehyde solution for Fluorometric TdT-mediated dUTP Nick-End labeling (TUNEL) assay. Briefly, the cells were permeabilized in 0.2% Triton X-100 solution for 5 min. DNA strand breaks were then labeled with fluorescein-12-dUTP in TdT incubation buffer at 37°C for 1 hr. The samples were then counterstained with 1 μg/ml PI in PBS, which binds to the A-T rich regions of DNA. Localized green fluorescence of apoptotic cells (fluorescein-12-dUTP) in a red background (PI) was detected by fluorescence microscopy. The percentage of apoptotic cells was obtained by dividing the number of cells with green fluorescence by the total number of cells counted. A minimum of 300 cells were counted for each condition.

Results

VEGF AS-ODNs Design and Assessment

Since VEGF has multiple isoforms resulting from alternative splicing (Figure 3-1), AS-ODNs design was targeted at the common region of all isoforms, regions around the AUG start codon. After screening several different designs, AS-ODNs V515 which is complimentary to the 5'UTR region just up-stream of the AUG start codon of the VEGF gene, was found to be most effective. The results showed that after 24 hr treatment with 1 μM VEGF AS-ODNs (V515) delivered by cationic liposome (DOTAP:DOPE), the medium VEGF levels were significantly reduced from a normal of 850 pg/ml/10^6 cells to 250 pg/ml/10^6 cells ($p<0.05$, student's *t*-test) (Figure 3-2). This antisense effect was sequence, and target region specific. Treating Caki-1 cells with liposome vehicles

(DOTAP: DOPE) or control scramble ODNs did not affect VEGF levels. Similarly, treatment with sense or inverted sequence ODNs failed to reduce VEGF expression.

Continued exposure of Caki-1 cells to the VEGF AS-ODNs (V515) resulted in a constant repression of VEGF in the culture medium (Figure 3-3). However, if the culture medium containing V515 was replaced with fresh medium 24 hr later, the VEGF levels in the Caki-1 cell medium gradually recovered, and reached about ~80% of that found in the untreated Caki-1 cell medium in about 7 days (Figure 3-3).

This repression of VEGF expression by V515 was also dose dependent (Figure 3-4). For example, a 24 hr treatment with 0.5 μM, reduced the medium VEGF level to 56% of control ($p<0.05$, student's *t*-test) whereas a 1 μM dose down-regulated the VEGF level to 22% of control ($p<0.05$, student's *t*-test).

VEGF mRNA levels in different AS-ODNs treatment groups also were determined (Figure 3-5). The results indicated a marked inhibition of VEGF mRNA after treatment with V515 which was absent in cells treated with scramble control ODNs. This result indicated that RNase H plays an important role in the function of V515.

BFGF AS-ODNs Design and Assessment

Alternative translation utilizing CUG start codons other than the AUG start codon leads to different isoforms bFGF gene products (Figure 3-6). In order to target all the bFGF isoforms using the same AS-ODNs sequence, the AS-ODNs were designed to target the common regions of all isoforms, especially around the AUG start codon. Effective AS-ODNs sequences against bFGF were identified and Caki-1 cells treated with them showed bFGF levels significantly lower than those normally observed (720 pg/ml/10^6 cells) (Figure 3-7). This effect was sequence and target region specific. The

AS-ODNs complimentary to the start codon (AUG) region (B460) was found to be the most effective. For example, the cellular bFGF level of B460 treated Caki-1 cells was found to be about 41% of that found in control or untreated cells ($p<0.05$, student's *t*-test). By comparison, the AS-ODNs complimentary to the 3' UTR (B931) or coding region (B471) were less effective at down regulating bFGF expression (57% and 65% of control values respectively, $p<0.05$, student's *t*-test). Since B460 had the most prominent inhibitory effect, it was used in all subsequent studies. Treating Caki-1 cells with control scramble ODNs or liposome vehicles did not affect bFGF levels in Caki-1 cells. Similarly, treatment with sense or inverted sequence ODNs failed to reduce bFGF expression. This inhibitory effect was also found to be AS-ODNs dose dependent (Figure 3-8). While a low dose of 0.5 μM B460 reduced the cellular bFGF level to about 80% of control, a high dose of 5 μM B460 led to a reduction by 65%.

BFGF mRNA levels in different PS-ODNs treatment groups also were determined (Figure 3-9). The results indicated a marked inhibition of bFGF mRNA after treatment with B460 which was absent in cells treated with scramble ODNs. Again, this suggests a role for RNase H in the efficacies of bFGF AS-ODNs B460.

Because FGF can have mitogenic effects in renal cells (Gospodarowicz et al., 1986; Issandou and Darbon, 1991), the influence of antisense and control ODNs treatment on Caki-1 cell growth was investigated. Control ODNs or liposome vehicles showed no effect on Caki-1 cell growth (Figure 3-10). In contrast, Caki-1 cell growth was significantly inhibited by AS-ODNs targeted against different regions of bFGF mRNA. B460 was found to be the most effective while AS-ODNs targeting the 3' UTR (B931) or coding region (B471) showed less cell growth inhibition. When comparing the data of

Figures 3-7 and 3-10, it also is apparent that the extent of Caki-1 cell growth inhibition by different AS-ODNs was closely related to their potency in down regulating bFGF expression.

In order to gain a better understanding of the underlying mechanisms of the observed growth inhibitory effect, FGF receptor expression was determined in Caki-1 cells (Figure 3-11). It was found that 3 out of 4 FGF receptors involved in the bFGF signal transduction pathway were expressed by Caki-1 cells, indicating that bFGF may play an autocrine role in Caki-1 cells. Additional studies indicated that B460 treatment had small but significant effects on Caki-1 apoptosis and cell cycle distribution (Figures 3-12 and 3-13). However, clonogenicity studies showed no significant difference between B460 treated and control cells indicating that B460 treatment had no direct cell killing effect on Caki-1 cells (data not shown). These findings suggest that blocking the bFGF signal transduction pathway may affect Caki-1 cell growth through cell cycle inhibition and induction of apoptotic cell death.

Discussion

Although dramatic advances have been made in the treatment of cancer, the development of efficacious anticancer agents still lags behind the rapid strides in our understanding of cancer biology, especially with the advent of molecular biology. The continued progress in our knowledge of the biology of neoplasm and in the identification, cloning and sequencing of genes critical to tumor cell function permits the exploitation of this information to develop specific agents that may directly modulate the function of these genes or their protein products. One methodology that takes direct advantage of

molecular sequencing data involves the use of antisense oligonucleotides (Ho and Parkinson, 1997).

The antisense-mediated gene inhibition was first introduced in 1978 by Stephenson and Zamecnik (Zamecnik and Stephenson, 1978; Stephenson and Zamecnik, 1978). The underlying concept is relatively straightforward: the use of a sequence, complementary by virtue of Watson-Crick basepair hybridization, to a specific mRNA can inhibit its expression and then induce a blockade in the transfer of genetic information from DNA to protein.

The selection of an appropriate target sequence is the first step in the process of AS-ODNs drug development. As a matter of fact, the hybridization between AS-ODNs and the target mRNA, which has a particular three dimensional shape resulting from secondary and tertiary structures, depends on the accessibility of the target sequence. Only limited stretches of mRNA sequences are actually available for heteroduplex formation with AS-ODNs. Still there is no sure way to determine *a priori* which AS-ODNs sequence would work best (Cohen, 1989;Woolf et al., 1992; Brysch and Schlingensiepen, 1994). The region surrounding the start codon (AUG) is probably the most popular target, followed by 5'UTR, coding regions or splicing sites. In the present studies, AS-ODNs sequence design and selection focused mainly on the start codon region of VEGF and bFGF genes. This region is also common to all isoforms of these two growth factors (Figure 3-1 and 3-6).

The most commonly used AS-ODNs are 18-20 bases in length. According to statistical calculations, a particular sequence of 13 bases in RNA and of 17 bases in DNA should be found only once in the entire human genome, thus representing unique

elements within the cell (Helene and Toulme, 1990). It also has been found that the activities of AS-ODNs increased with AS-ODNs length, but the increased thermodynamic stability of hybridization observed with AS-ODNs binding to non-target mRNA sequences that may be similar, but not identical to the target sequence, results in reduced specificity (Monia et al., 1992). Thus, an AS-ODNs length of 20-mer is usually considered optimal and therefore was used in the present studies for all the designed AS-ODNs. To ensure the specificity of the target sequence, all designed AS-ODNs sequences were checked for global sequence comparison using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). Only sequences specific for the VEGF/bFGF gene and having at least 4 miss-match bases with other genes were used. In addition, polyguaosine (GGGG), which is known to exert non-antisense effects was avoided (Benimetskaya et al., 1997). Lastly, all the AS-ODNs designed were examined for secondary structures such as hairpins, self-dimers, and cross-dimers using Netprimer (PREMIER Biosoft International, Palo Alto, CA).

Since cells contain a variety of exo- and endonucleases that can degrade ODNs, nucleotide modifications have been made to make the AS-ODNs more resistant to nuclease digestion than the native ODNs that have phosphodiester linkages in their backbones. The most widely explored analogues have been the phosphorothioates, in which one of the nonbridging oxygen atoms in each internucleoside phosphate linkage is replaced by a sulfur atom. This modification is easily adapted to automated synthesis and confers metabolic stability because its resistance to degradation by DNase (Stein et al., 1988). In addition, these analogues retain water solubility and permit RNase H mediated

hydrolysis of the target mRNA strand. This modification has been successfully used in a variety of investigations (Galderisi et al., 1999; Ho and Parkinson, 1997; Crooke, 1998). Based on these favorable properties and extensive information available for its application, phosphorothioate modification was used in the current investigations of VEGF/bFGF AS-ODNs.

AS-ODNs may exert biological activity through a variety of mechanisms. Although some of the mechanisms of inhibition have been characterized, rigorous proof for others is still frequently lacking. Two classes of AS-ODNs can be discerned: (a) the RNase-H dependent ODNs, which induce the degradation of mRNA; and (b) the steric-blocker ODNs, which physically prevent or inhibit the progress of splicing or translational machinery (Crooke, 1992; Dias N and Stein, 2002).

The most commonly implicated antisense mechanisms relate to RNase H mediated hydrolysis of the target mRNA. This is also the case for most of the antisense drugs investigated in the clinic. RNase H is a ubiquitous enzyme that hydrolyzes the RNA strand of the RNA/DNA hybrid. Thus the binding of AS-ODNs to its target mRNA may induce digestion of the message. AS-ODNs assisted RNase H dependent reduction of target RNA expression can be quite efficient, reaching 80-95% down-regulation of protein and mRNA expression (Dias N and Stein, 2002). Furthermore, in contrast to the steric-blocker ODNs, RNase H dependent ODNs can inhibit protein expression when a much wider region of the mRNA is targeted. Thus, unlike most steric-blocker ODNs that are efficient only when targeted to certain 5' UTR or AUG start codon regions, RNase H dependent AS-ODNs can exert effects when targeted to widely separated areas in the coding region as well (Dean and McKay, 1994; Larrouy et al., 1992). In the present

studies, mRNA levels of VEGF and bFGF were significantly down-regulated after the AS-ODNs treatment (Figures 3-5 and 3-9). These findings suggest that current AS-ODNs function mainly through the RNase H mechanism.

Successful design and evaluation of AS-ODNs relies on their efficient delivery of into the cytoplasm, where they can exert their antisense effect. A cationic liposome (DOATP:DOPE) based delivery system was used to deliver AS-ODNs in the present studies. The evaluation and optimization of this delivery system has been discussed in detail in Chapter 2.

Following the aforementioned guidelines in AS-ODNs design and selection, effective AS-ODNs against VEGF and bFGF have been identified. Treatment of Caki-1 cell with the AS-ODNs led to significant repression of VEGF and bFGF expression levels (Figures 3-2 and 3-7). These effects were ODNs sequence specific, dose dependent and could be achieved at low, non-toxic doses. These results indicate that AS-ODNs can be used to efficiently modulate the specific target gene expressions.

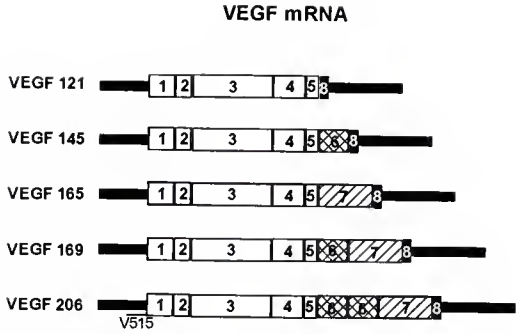


Figure 3-1. VEGF mRNA structure showing all isoforms derived from alternative splicing.

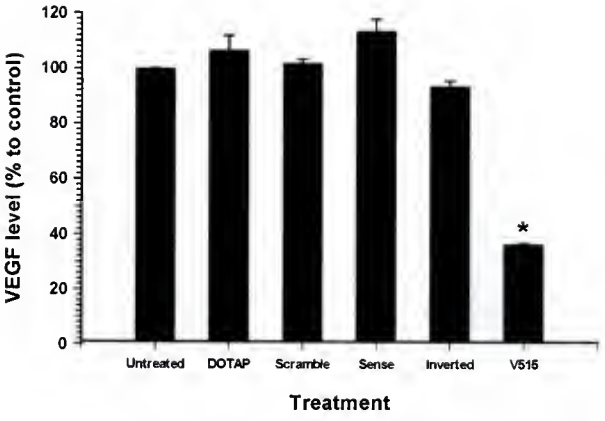


Figure 3-2. VEGF levels in culture medium of Caki-1 cells treated with different AS-ODNs. Caki-1 cells were untreated, treated with vehicle (DOTAP) only, treated with 1 μM control ODNs or VEGF AS-DONs (V515) for 24 hr. The 100% VEGF expression level of the untreated group corresponds to ~800 pg/ml/10^6 cells. Each bar represents the mean ± S.E. of 3 independent experiments. The star indicates a statistically significant difference from the untreated group ($p<0.05$, student's *t*-test).

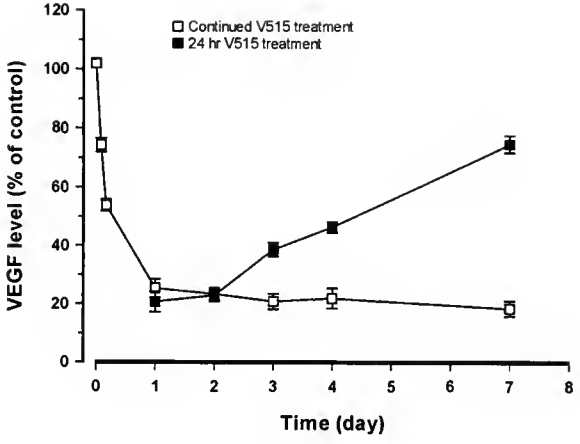


Figure 3-3. VEGF levels in the culture medium of Caki-1 cells at different times after V515 treatment. Media containing V515 were either unchanged (□) or replaced with fresh medium after 24 hr (■). Each datum point represents the mean ± S.E. of 3 independent experiments.

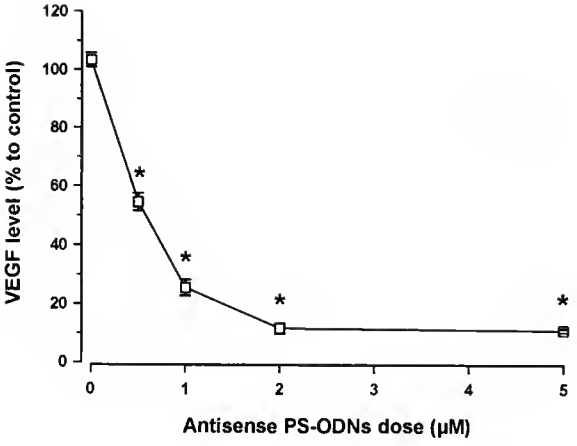


Figure 3-4. VEGF levels in culture medium of Caki-1 cells after treatment with different doses of V515. The 0 dose represents the Caki-1 cells treated with 5 μM control scramble ODNs. Each datum point represents the mean ± S.E. of 3 independent experiments. Stars indicate statistical significance compared to the control ODNs treated ($p<0.05$, student's *t*-test).

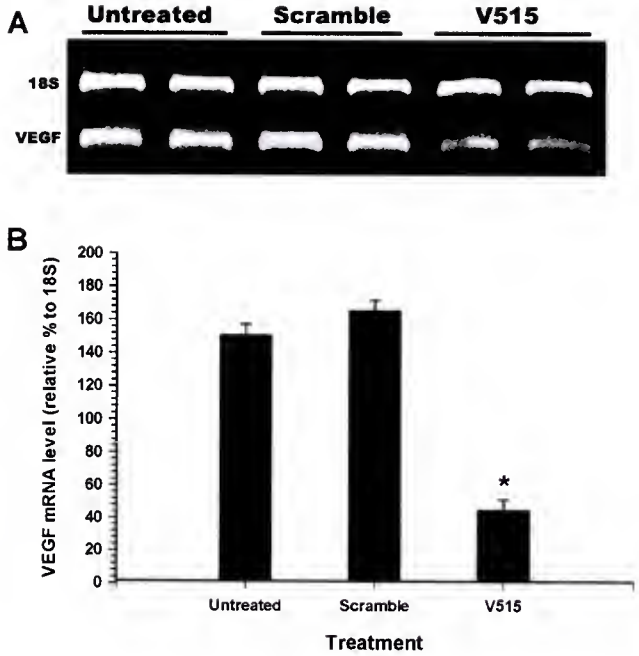


Figure 3-5. Message RNA levels in Caki-1 cells either untreated, treated with a 1 μM dose of scramble ODNs or VEGF AS-ODNs (V515). A) Representative relative RT-PCR results; B) Relative VEGF mRNA levels of Caki-1 cells after the treatment. The star indicates a statistical significance compared to untreated control ($p<0.05$, student's *t*-test).

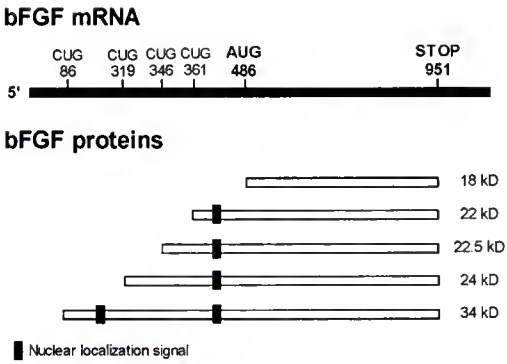


Figure 3-6. Message RNA structure and protein products of bFGF gene. Modified from Okada-Ban (Okada-Ban et al., 2000).

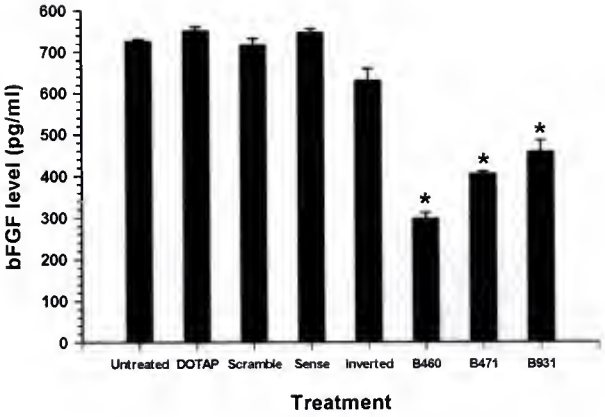


Figure 3-7. Cellular bFGF levels of Caki-1 cells after AS-ODNs treatment. Caki-1 cells were either untreated, treated with liposome alone, 1 μM control ODNs or bFGF AS-ODNs for 24 hr. Each bar represents the mean ± S.E. of 3 independent experiments. Stars indicate statistically significant difference from the untreated control group ($p<0.05$, student's *t*-test).

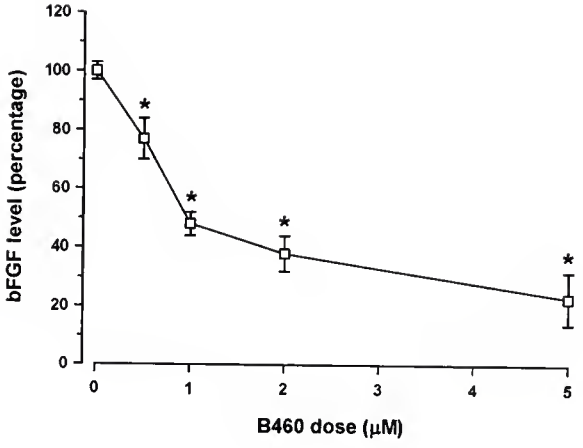


Figure 3-8. Cellular bFGF levels in culture medium of Caki-1 cells after treatment with different doses of B460. The 0 dose Caki-1 cells treated with 5 μM control scramble ODNs. Each datum point represents the mean ± S.E. of 3 independent experiments. Stars indicate statistically significant differences compared to the control ODNs treated group ($p<0.05$, student's *t*-test).

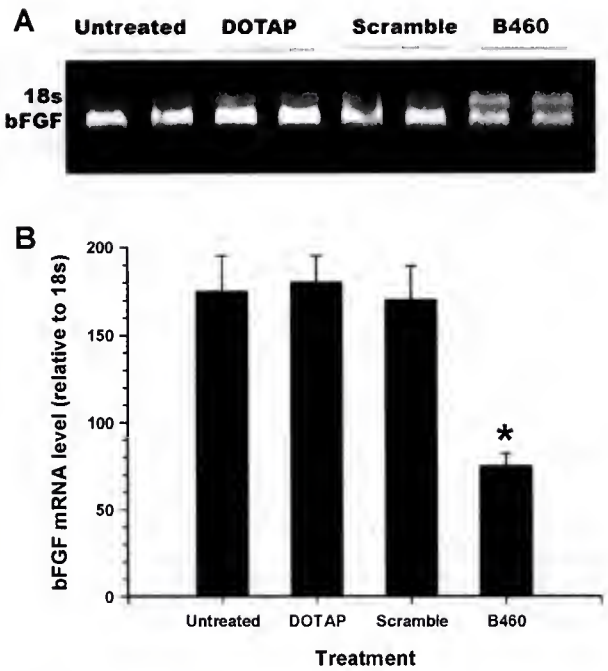


Figure 3-9. Message RNA levels in Caki-1 cells either untreated, treated with a 1 μM dose of scramble ODNs or bFGF AS-ODNs (B460). A) Representative relative RT-PCR results; B) Relative bFGF mRNA levels of Caki-1 cells after the treatment. The star indicates a statistically significant difference compared to the untreated control group ($p<0.05$, student's *t*-test).

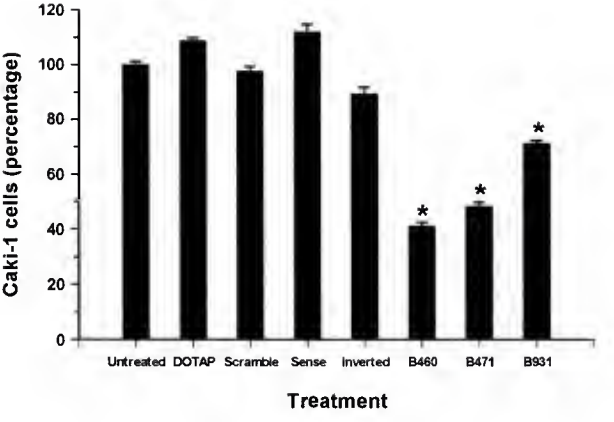


Figure 3-10. Effect of AS-ODNs treatment on Caki-1 cell growth. Caki-1 cells were either untreated, treated with liposome alone, 1 μM control ODNs or bFGF AS-ODNs for 4 days and the number of cells determined. Each bar represents the mean ± S.E. of 3 independent experiments. Stars indicate statistically significant differences compared to untreated control group ($p<0.05$, student's *t*-test).

Figure 3-11. FGF receptor expression in Caki-1 cells. RT-PCR results of FGF receptors 1-4 and control 18s expression in Caki-1 cells.

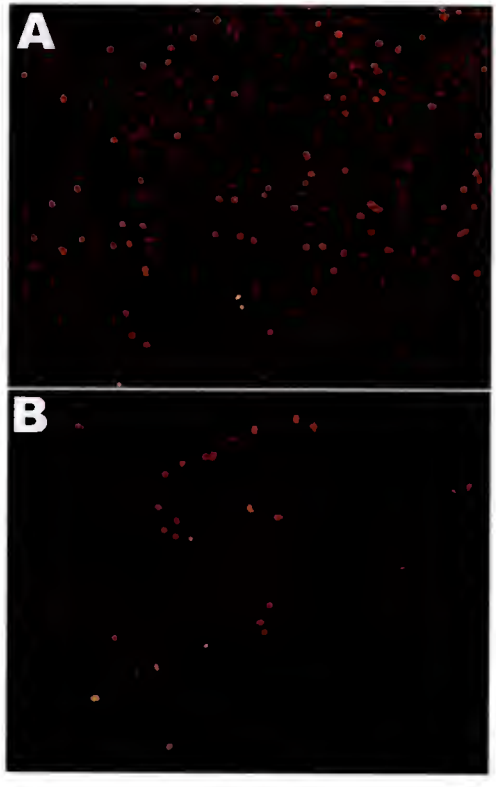


Figure 3-12. Apoptotic cell death in Caki-1 cells either untreated or treated with bFGF AS-ODNs. A) Representative picture of untreated Caki-1 cells stained with DeadEnd™ Fluorometric TUNEL System; B) representative picture of Caki-1 cells after treatment with B460 stained with DeadEnd™ Fluorometric TUNEL System.

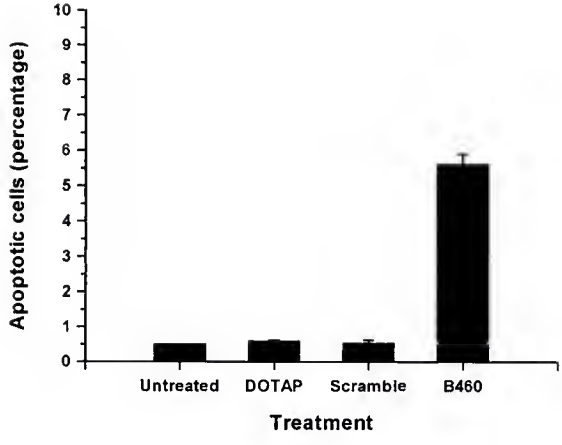


Figure 3-13. Apoptotic cell death in Caki-1 cells after AS-ODNs treatment. Caki-1 cells were either untreated, treated with liposome alone, treated with 1μM scramble control ODNs or bFGF AS-ODNs. Each bar represents the mean ± S.E. of 3 independent experiments. The star indicated a statistically significant difference from the untreated control group ($p<0.05$, student's *t*-test).

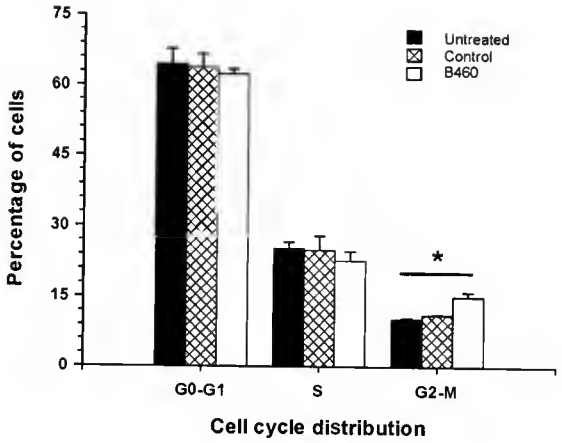


Figure 3-14. Effect of bFGF AS-ODNs treatment on the cell cycle distribution of Caki-1 cells. Each bar represents the mean ± S.E. of 3 independent experiments. Stars indicate statistical significant differences compared to the untreated control group ($p<0.05$, student's *t*-test).

CHAPTER 4
ANTI-ANGIOGENIC EFFICACY STUDIES

Introduction

The greater understanding of the process of tumor angiogenesis, coupled with the notion that tumors require a blood supply to grow and metastasize, has fueled the research for strategies that block or disrupt the angiogenic process. Moreover, because normal vascular endothelial cells turn over so slowly, conventional wisdom suggests that an anti-angiogenic approach to cancer therapy should offer improved efficacy and reduced toxicity, with much less potential for drug resistance.

Angiogenesis is a complex process with multiple, sequential and interdependent steps (Fidler, 1999). This complexity creates many potential targets for inhibition. Key characteristic of the immature vasculature of tumors have allowed the development of several categories of anti-angiogenic agents (Kerbel, 2000). Preclinical studies have identified agents that (i) inhibit endothelial cell activation (ii) inhibit endothelial proliferation/migration (iii) inhibit basement membrane degradation and (iv) inhibit integrin receptor activation.

Angiogenesis can be qualitatively and quantitatively measured in a large variety of *in vitro* and *in vivo* model systems. As mentioned before the angiogenic cascade can be dissected into different sequential steps so that can be studied separately *in vitro*. Research has mainly focused on the proliferation and migration of endothelial cells as key elements for angiogenic potential *in vitro*. For this research, different endothelial cell sources can be utilized. For human tumor research most laboratories make use of

HUVECs. Although readily available, a major advantage, the major drawback of these cells is their macrovascular origin, which makes them less suitable for studies on angiogenesis, a microvascular process. In recent years, microvascular endothelial cells derived from different organs have been established and become commonly available. Protocols for isolating purified tumor endothelial cells also have been developed (St Croix et al., 2000).

Assays to study proliferation of endothelial cells are based on cell counting, radio-labeled thymidine incorporation, or on colorimetric assays for measurement of mitochondrial activity. Detection of cell death also is used to determine cell growth effects. To measure endothelial cell migration, Boyden chambers are primarily used. Though an easier system based on wounding of a confluent monolayer of endothelial cells and measuring wound width or invading cells as a function of time is also available, it maybe physiologically less relevant to tumor angiogenesis. In the present studies, we established a co-culture system based on modified boyden chambers to evaluate endothelial cell proliferation and migration potential (Figure 4-1 and 4-4). In order to mimic the *in vivo* interaction of tumor and endothelial cells, co-cultured tumor cells were the primary source of pro-angiogenic growth factors for the endothelial cells.

The advantage of the *in vitro* assays is clearly the control that can be exerted over selected parameters. However the angiogenic cascade consists of multiple steps in their entirety, *in vivo* investigations are needed. The most frequently used *in vivo* assay systems are the chicken chorioallantoic membrane assay (Nguyen et al., 1994a), the corneal pocket assay (Conrad et al., 1994), transparent chamber preparations such as the dorsal skin-fold chamber (Algire G.H., 1943; Lichtenbeld et al., 1998), the cheek pouch

window (Shubik et al., 1976) and polymer matrix implants (Mahadevan et al., 1989; Plunkett and Hailey, 1990). A simpler system using intradermal implantation of tumors cells is also available to study the tumor angiogenic process *in vivo* (Sidky and Auerbach, 1976). This assay has been validated for evaluating tumor-induced angiogenesis *in vivo* using a variety of different tumor models and treatment interventions (Lindner and Borden, 1997; McMillan et al., 1999; Danielsen and Rofstad, 1998) and was applied in the present studies .

Through the utilization of both the co-culture system *in vitro* and the intradermal angiogenesis assay *in vivo*, objective and reasonable assessments of the anti-angiogenic efficacy of treatment interventions can be achieved. In the present studies, the anti-angiogenic efficacy of VEGF and bFGF AS-ODNs treatments were evaluated using these *in vitro* and *in vivo* models.

Materials and Methods

Cell Culture

The clear cell RCC cell line Caki-1 was a gift from Dr. Susan Knox (Stanford University, CA). Caki-1 cells were grown in Dulbecco's modified minimum essential medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY), 1% penicillin-streptomycin (Invitrogen, Grand Island, NY) and 1% 200 mmol/L L-glutamine (Invitrogen, Grand Island, NY).

The mouse heart endothelial cell line (MHE) was a gift from Dr. Robert Auerbach (University of Wisconsin, WI). MHE cells were grown in DMEM supplemented with 10% heat inactivated FBS, 1% penicillin-streptomycin and 1% 200 mmol/L L-glutamine. Human microvascular endothelial cell from the lung (HMVEC-L) cells were obtained

from Clonetics (San Diego, CA). HMVEC-L cells were grown in EBM-2-MV (Clonetics, San Diego, CA) supplemented with 5% FBS.

Antisense Phosphorothioate Oligodeoxynucleotides (AS-ODNs)

Antisense and control ODNs (20-mers) were custom synthesized by Geno Mechanix (Alachua, FL). The entire backbone of all ODNs was phosphorothioate modified. AS-ODNs V515 was complementary to 5' UTR just up-stream of the translation start site (AUG codon) of VEGF mRNA: 5' - CTC ACC CGT CCA TGA GCC CG - 3'. Scramble sequence: 5' – CAC CCT GCT CAC CGC ATG GC - 3'; sense sequence: 5' - CGG GCT CAT GGA CGG GTG AG - 3' and an inverted sequence: 5'-GCC CGA GTA CCT GCC CAC TC - 3', were used as ODNs controls. AS-ODNs B460 was complementary to the translation start site (AUG codon) of bFGF mRNA: 5' TCC CGG CTG CCA TGG TCC CT 3'. Scramble sequence: 5' GCC TGG ACC CTG GCT CTC TC 3'; sense sequence: 5' AGG GAT GGC TGC CGG GA 3' and an inverted sequence: 5' TCC CTG GTA CCG TCG GCC CT 3' were used as controls. All ODNs were suspended in sterile and endotoxin free water at a concentration of 1 mM, aliquoted and stored at -20°C.

DOTAP:DOPE Liposome Preparation

Cationic liposomes were prepared using the method described by Tang (Tang and Hughes, 1999). Briefly, cationic lipid 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) was dissolved in chloroform and mixed with a helper lipid 1,2-dioleoyl-3-sn-phosphatidylethanolamine (DOPE) (Avanti Polar-Lipids, Alabaster, Al) at a molar ratio of 1:1. The mixture was evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The resulting lipid film was dried by nitrogen for an

additional 10 min to evaporate any residual chloroform. The lipid film was re-suspended in sterile water to a final concentration of 1 mg/ml based on the weight of cationic lipid. The resultant mixtures were shaken in a water bath at 35°C for 30 min. The suspensions then were sonicated using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) for 1 min at room temperature to form homogenized liposomes. The particle-size distribution of liposomes was measured using a NICOMP 380 ZLS instrument (Santa Barbara, CA). The average particle diameter was 144.0 ± 77.0 nm. Liposomes were stored at 4°C and used within 3 months.

Co-culture Assay

Transwell (Corning, Corning, NY) 6-well dishes with a membrane pore size of 0.4 μM were used. Caki-1 cells were seeded at 5×10^4 in the transwell inserts and MHE or HMVEC-L cells were plated at 5×10^4 per well in the 6-well dishes and allowed to attach overnight. The Caki-1 cell medium then was replaced with serum free medium containing 1 μM V515 or B460 AS-ODNs or control ODNs delivered with liposome (DOTAP:DOPE). After a 5 hr of treatment, medium containing 10% heat inactivated FBS was added to yield a final FBS concentration of 2.5%. The transwells containing treated Caki-1 cells were assembled with 6-well dished containing MHE and HMVEC-L cells and incubated at 37°C for 72 hr at which time the numbers of MHE or HMVEC-L cells were determined by haemocytometer count (Figure 4-1).

Migration Assay

Caki-1 cells were set at 1×10^5 per well in 24-well dishes and allowed to attach overnight. The Caki-1 cells then were treated with 1 μM V515 or B460 AS-ODNs or control ODNs for 24 hr. HTS FluoroBlok inserts (Becton Dickinson, Franklin Lakes, NJ)

with a pore size of 8.0 μm were assembled into the 24-well dish with the Caki-1 cells. MHE or HMVEC-L cells were grown in T-150 flasks to about 80% confluence. The endothelial cells were stained in medium containing 10 μg/ml Di-1 (Molecular Probes, Eugene, OR) for 24 hr, washed 4 times with PBS, collected and added into the FluoroBlok inserts (5×10^4 MHE or HMVEC-L) and incubated for another 24 hr. The number of migrated endothelial cells then was determined by direct measurement of the fluorescence in the bottom well using a CytoFluor 4000 plate reader (Perceptive BioSystems, St. Paul, MN). (Figure 4-4)

Intradermal Angiogenesis Assay

Caki-1 cells were treated with AS-DONs for 5 hr *in vitro* as described before. The cells were then collected and inoculated intradermally (5×10^4) in a volume of 10 μl at 4 sites on the ventral surface of nude mice. One drop of 0.4% trypan blue was added to the cell suspension before injection, which making it lightly colored, simplified subsequent location of the sites of injection. Three days later the mice were killed, the skin was carefully separated from the underlying muscle and the number of vessels counted using a dissecting microscope (Sidky and Auerbach, 1976). Scoring of all of the reaction areas was carried out at the same magnification (5x) and only vessels readily detected at this magnification were counted. The sites of injection, recognized by local swelling and blue staining, were exposed by carefully removing fat or other tissue covering the area. All vessels that touched the edge of the tumor inoculates were counted. All the animals in the experiments were pre-coded and vessel counts in each animal were scored twice. The resultant data points for each treatment group were pooled for statistical analysis (Wilcoxon rank sum test).

Results

Anti-angiogenic efficacy of VEGF/bFGF AS-ODNs treatments was first evaluated *in vitro*. The transwell co-culture system was used to examine the effect of AS-ODNs treatment of Caki-1 cells on the proliferation of co-cultured endothelial cells (Figure 4-1). This setting allowed the constant exchange of growth factors without direct tumor-endothelial cell-cell interaction and mimicked the paracrine interaction between tumor and endothelial cells. Since a human RCC tumor cell line and xenograft in nude mice were used in these studies, both human (HMVEC-L) and mouse (MHE) endothelial cells were studied. Caki-1 tumour cells were grown in transwells with 0.4 μm membrane pores. The effects of pretreating Caki-1 tumor cells with VEGF or bFGF AS-ODNs on endothelial cell proliferation then were determined (Figure 4-2 and 4-3). The results showed that compared to untreated Caki-1 cells, Caki-1 cells pre-treated with V515 or B460 significantly inhibited both HMVECV-L and MHE cell proliferation. Once again, treating Caki-1 cells with a variety of control PS-ODNs had no effect on HMVEC-L or MHE cell growth.

To test whether a reduction in VEGF or bFGF expression by tumor cells could affect endothelial cell migration, HMVEC-L or MHE cells were stained with 10 μg/ml Di-I for 24 hours and added into Fluoroblok inserts placed into 24 well dishes containing Caki-1 tumour cells treated with V515. The number of pre-labelled endothelial cells which migrated through the 8 μm pore size membranes in a 24 hr period were quantified by determining the fluorescence intensity in the bottom well (Figure 4-4). The results showed (Figure 4-5) that 24 hr after co-culturing the two cell populations ~45% ($p<0.05$, student's *t*-test) and 37% ($p<0.05$, student's *t*-test) fewer MHE or HMVEC-L

cells respectively migrated through the membrane in the presence of V515 treated Caki-1 cells compared to untreated or scramble control AS-ODNs treated Caki-1 cells. Similarly, ~37% ($p<0.05$, student's *t*-test) and ~33% ($p<0.05$, student's *t*-test) fewer MHE or HMVEC-L cells respectively migrated through the membrane in the presence of B460 treated Caki-1 cells compared to untreated or scramble treated control AS-ODNs (Figure 4-6).

Although, the *in vitro* studies indicated that treating Caki-1 tumor cells with VEGF or bFGF mRNA targeted AS-ODNs down-regulated VEGF/bFGF protein production sufficiently to affect the proliferation and migration of endothelial cells, it was important to demonstrate that such treatments also could affect Caki-1 cell induction of angiogenesis *in vivo*. To examine this possibility Caki-1 cells that had been treated with V515, B460 or control ODNs were injected intradermally and the number of vessels induced were counted 3 days later (Figure 4-7). While untreated Caki-1 cells and control ODNs treated Caki-1 cells had very similar angiogenic potency *in vivo* (both groups induced ~44-46 new vessels in the assay period), the angiogenic potential of Caki-1 cells that had been pre-treated with VEGF AS-ODNs (V515) was found to be significantly impaired; only ~25.5 ($p<0.05$, Wilcoxon rank sum test) new blood vessels were observed. Similarly, bFGF AS-ODNs (B460) treated tumor cells also induced less vessels, ~27 new blood vessels ($p<0.05$, Wilcoxon rank sum test). More importantly, the most significant inhibition of formation of new blood vessels was observed when the Caki-1 cells were treated with both V515 and B460, only ~20 new vessels developed ($p<0.05$, Wilcoxon rank sum test).

Discussion

Anti-angiogenesis treatment strategies represent a new approach to cancer management. Given that solid tumors cannot progress effectively without the generation of new blood vessels, various tacks have been taken to interfere tumor angiogenesis. One possible target which has received considerable attention is the pro-angiogenic factor VEGF. VEGF can induce endothelial cell proliferation and migration *in vitro* (Soker et al., 1997; Hanahan and Folkman, 1996a; Hanahan and Folkman, 1996b) and angiogenesis *in vivo* (Leung et al., 1989; Plate et al., 1992a; Plate et al., 1992b). Its expression level has been associated with a variety of tumors and correlated to treatment outcome (Gasparini et al., 1997; Maeda et al., 1996a; Maeda et al., 1996b). Basic fibroblast growth factor (bFGF) is another important pro-angiogenic factor (Yoshida et al., 1996; Hoying and Williams, 1996). It also has been found to associated with different tumors and correlated to treatment outcome, especially RCC (Nguyen et al., 1994b; Nanus et al., 1993; Miyake et al., 1996). Antisense oligodeoxynucleotide technology provides an approach for inhibiting gene expression with target specificity as a particular advantage (Stein and Cheng, 1993; Engelhard, 1998a; Engelhard, 1998b). Effective AS-ODNs against VEGF and bFGF have been identified and tested *in vitro* (Chapter 3).

To evaluate the VEGF/bFGF suppression through the use of AS-ODNs on Caki-1 tumor angiogenesis, both *in vitro* and *in vivo* efficacies were studied. The co-culture system, which allows constant exchange of growth factors between tumor and endothelial cells was used to study the treatment on endothelial proliferation. The tumor cells also served as primary source of pro-angiogenic growth factors for endothelial cells. This provides a setting that closely mimics the *in vivo* situation. Significantly impaired

proliferation potential of both human and mouse microvascular endothelial cells were observed after VEGF/bFGF AS-ODNs treatment (Figure 4-2 and 4-3). In order to evaluate the AS-ODNs treatment on endothelial cell migration potential, a very similar co-culture system (FluoroBlok system) utilizing 24 well dishes and larger pore sized membranes (8 μM) was used. The results showed that suppression of VEGF/bFGF expression by AS-ODNs was sufficient to inhibit the migration of endothelial cells in response to pro-angiogenic growth factors produced by tumor cells (Figure 4-5 and 4-6).

VEGF/bFGF AS-ODNs treatment of Caki-1 cells led to inhibition of endothelial cell proliferation and migration in both human and mouse microvascular endothelial cells (Figure 4-2, 4-3, 4-5 and 4-6) suggesting that such treatments should also exert their effects in a mouse model. In deed, the anti-angiogenic efficacy of VEGF/bFGF AS-ODNs was readily demonstrated *in vivo* using the intradermal angiogenesis assay (Figure 4-8).

These results not only support the role of VEGF and bFGF as important pro-angiogenic growth factors in Caki-1 cell induced angiogenesis, but also clearly suggest that inhibition of cancer cell VEGF or bFGF expression may ultimately impact tumor growth. The use of AS-ODNs against VEGF/bFGF was sufficient to illicit anti-angiogenic effects both *in vitro* and *in vivo*.

Taken together, these findings suggest that AS-ODNs targeted to VEGF and bFGF are effective in inhibiting Caki-1 tumor cell induced angiogenesis. They further implying that such a treatment strategy may have utility in the treatment of RCC.

Figure 4-1. Transwell co-culture system for evaluating VEGF/bFGF AS-ODNs treatment of Caki-1 cells on endothelial cell proliferation.

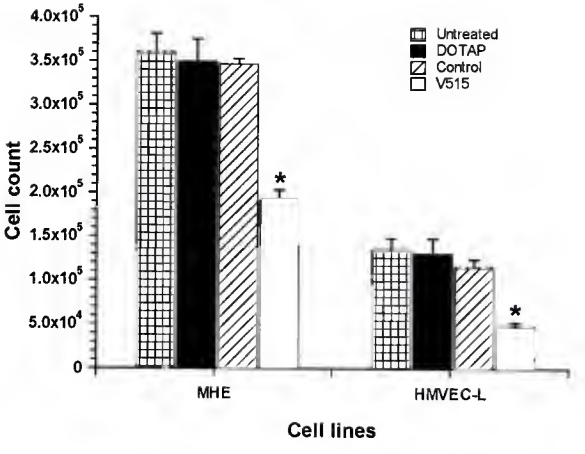


Figure 4-2. Treatment of Caki-1 cells with VEGF AS-ODNs on the growth of co-cultured endothelial cell. Caki-1 cells were either untreated, treated with liposome alone, treated with 1 μM scramble control ODNs or VEGF AS-ODNs. Each bar represents the mean ± S.E. of 3 independent experiments. Stars indicate statistical significance compared to untreated control group ($p<0.05$, student's *t*-test).

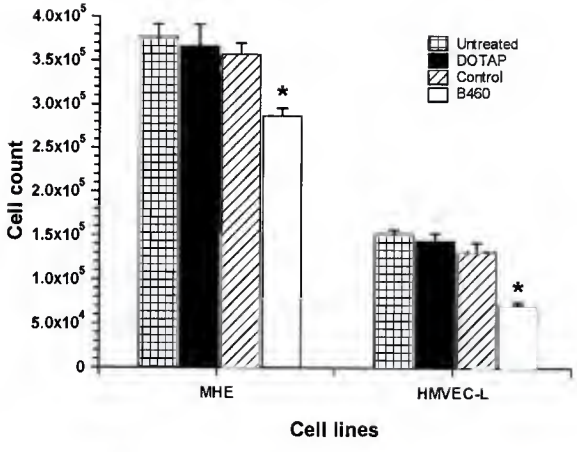


Figure 4-3. Treatment of Caki-1 cells with bFGF AS-ODNs on the growth of co-cultured endothelial cell. Caki-1 cells were either untreated, treated with liposome alone, treated with 1 μM scramble control ODNs or bFGF AS-ODNs. Each bar represents the mean ± S.E. of 3 independent experiments. Stars indicate statistical significance compared to untreated control group ($p<0.05$, student's *t*-test).

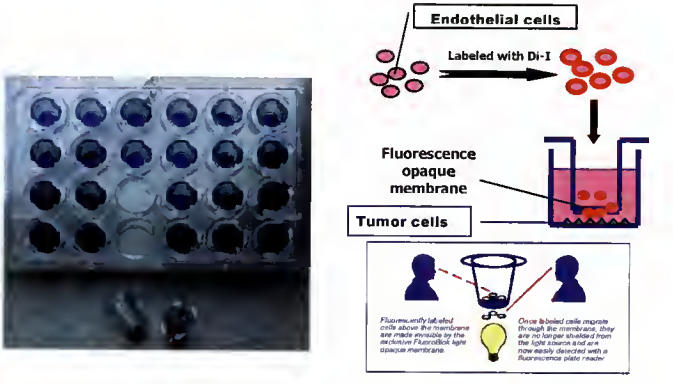


Figure 4-4. Transwell co-culture system for evaluating VEGF/bFGF AS-ODNs treatment of Caki-1 cells on the migration potential of endothelial cells.

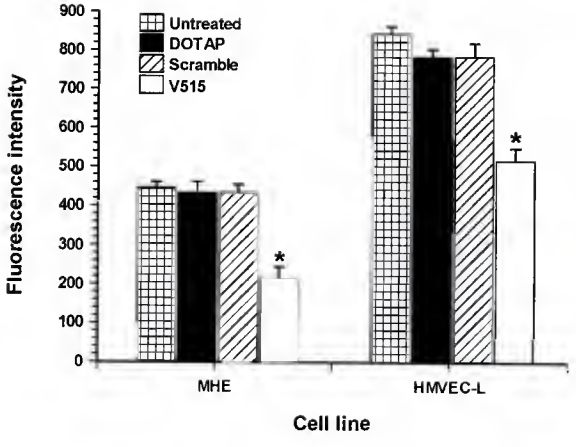


Figure 4-5. Treatment of Caki-1 cells with VEGF AS-ODNs on the migration potential of co-cultured endothelial cells. Caki-1 cells were either untreated, treated with liposome alone, treated with 1 μM scramble control ODNs or VEGF AS-ODNs. Each bar represents the mean ± S.E. of 3 independent experiments. Stars indicate statistical significance compared to untreated control group ($p<0.05$, student's *t*-test).

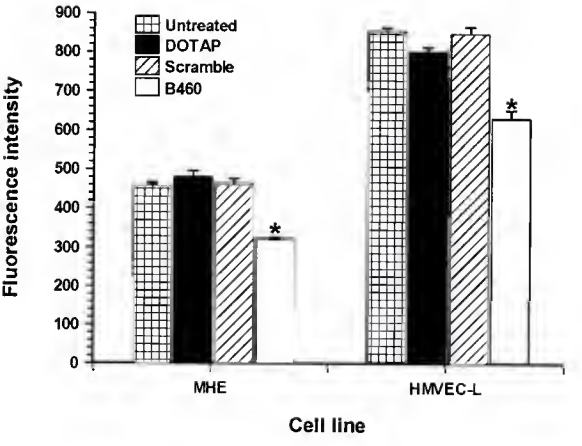


Figure 4-6. Treatment of Caki-1 cells with bFGF AS-ODNs on the migration potential of co-cultured endothelial cells. Caki-1 cells were either untreated, treated with liposome alone, treated with 1 μM scramble control ODNs or bFGF AS-ODNs. Each bar represents the mean ± S.E. of 3 independent experiments. Stars indicate statistical significance compared to untreated control group ($p<0.05$, student's *t*-test).

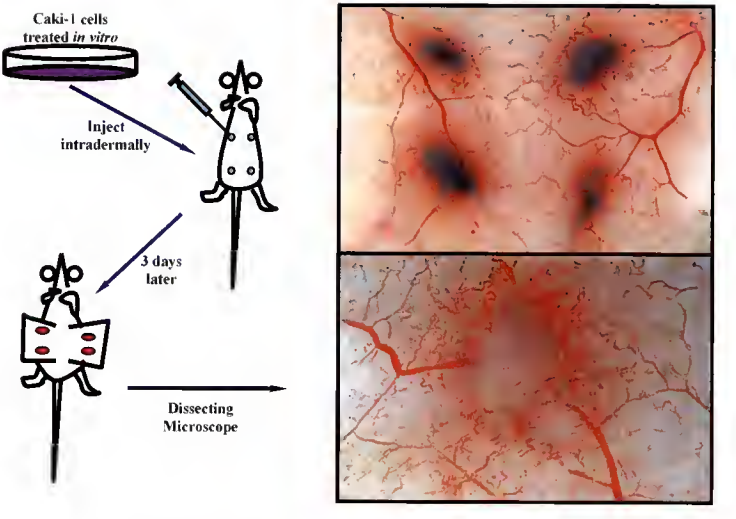


Figure 4-7. Intradermal angiogenesis assay. Caki-1 cells were treated *in vitro* with AS-ODNs and were then injected intradermally into the ventral surface of nude mice. Three days later, the skin was removed and the vessels induced were counted using a dissecting microscope. Right: representative pictures of tumor induced angiogenesis in an intradermal angiogenesis assay.

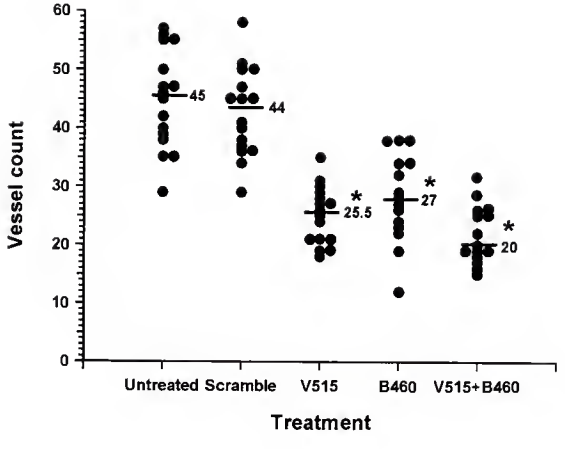


Figure 4-8. Number of blood vessels induced 3 days after injecting Caki-1 cells. Caki-1 cells were either untreated, or pre-treated with 1 μM scramble control ODNs, VEGF AS-ODNs, bFGF AS-ODNs or both. Each datum point presents one injection site. Bar represents the median of 16 injection sites in each group. Stars indicate statistical significance compared to untreated control group ($p<0.05$, Wilcoxon rank sum test).

CHAPTER 5
EFFICACY OF AS-ODNS TREATMENT IN CAKI-1 XENOGRAFTS

Introduction

Renal cell carcinoma (RCC) is the most common malignancy of the kidney in adults and accounts for about 3% of all adult malignancies (Jemal et al., 2002). Unless discovered at an early stage, at a time when it is a still a resectable neoplasm, RCC has a very unfavorable treatment outcome to conventional measures. Unfortunately, RCC is characterized by a lack of early warning signs, resulting in a high proportion of patients with metastases at diagnosis. As a consequence RCC remains fatal in nearly 80% of its patients (Tsui et al., 2000a; Tsui et al., 2000b; Lau et al., 2002; Delahunt et al., 2002).

Histopathologic evaluations of RCC reveal it to be a highly vascularized neoplasm demonstrating clear evidence of abundant angiogenesis and abnormal blood vessel development (Yoshino et al., 2000; Slaton et al., 2001). Not surprisingly, several studies have pointed to an important role for pro-angiogenic growth factors in RCC. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have often been implicated. Both factors have been shown to be expressed in renal cell carcinoma tissues and renal cell carcinoma cell lines (Mydlo et al., 1993; Gospodarowicz et al., 1986; Sato et al., 1999; Hemmerlein et al., 2001; McLaughlin and Lipworth, 2000; Ferrara and Keyt, 1997). Serum levels of VEGF and bFGF often are elevated in RCC patients (Nguyen et al., 1994b; Fujimoto et al., 1991; Wechsel et al., 1999; Tomisawa et al., 1999; Paradis et al., 2000) and renal cell carcinoma VEGF and bFGF mRNA levels have been reported to be much higher than those found in surrounding normal tissues

(Tricarico et al., 1999; Thelen et al., 1999; Tsuchiya et al., 2001; Eguchi et al., 1992). In addition, elevated serum/urine bFGF levels have been shown to associated with malignant progression and poor treatment outcome (Song et al., 2001; Jacobsen et al., 2000; Rasmuson et al., 2001; Edgren et al., 1999; Dosquet et al., 1997; Fujimoto et al., 1995; Plunkett and Hailey, 1990; Miyake et al., 1996; Nguyen et al., 1994a; Huang et al., 1996). Taken together, these findings suggest that VEGF and bFGF are the important factors involved in the angiogenic process of RCC.

Currently, there is great interest in anti-angiogenic therapies for the treatment of RCC. For example, interferon-α, a peptide known to have anti-angiogenic effects likely due to suppression of bFGF expression (Singh et al., 1995), has been shown to prolong survival in patients with RCC. Interleukin-12, a cytokine with immuno-regulatory and anti-angiogenic activity (Voest et al., 1995), also has demonstrated antitumor activity in RCC (Motzer et al., 1998). Other drugs developed principally as angiogenesis inhibitors and studied in RCC include the fumigillin analog TNP-470, thalidomide, and a monoclonal antibody to VEGF (Stadler et al., 1999; Chow et al., 1996).

AS-ODNs treatment is another effective measure to suppress the expression of pro-angiogenic factors. Previous studies have identified effective AS-ODNs sequences again VEGF and bFGF (Chapter 3), and their anti-angiogenic efficacy has been demonstrated *in vitro* and *in vivo* (Chapter 4). In the present investigations, AS-ODNs complementary to VEGF or bFGF mRNA were tested for their anti-tumor efficacy through systemic administration in a solid tumor model of RCC (Caki-1 xenografts).

Materials and Methods

Caki-1 Xenografts

Female nude mice (NCR, nu/nu), age 6 - 8 weeks were maintained under specific-pathogen-free conditions (University of Florida Health Science Center) with food and water supplied *ad libitum*. Initial Caki-1 xenografts were established by inoculating 5×10^6 Caki-1 tumor cells subcutaneously into the flanks of nude mice. Because of the rather long time for the xenografts to appear and grow, and the relatively low take rate, subsequent passages of Caki-1 tumors were achieved by implanting tumor pieces subcutaneously in the flanks of recipient animals. When the tumors reached a size ~200 mm^3, animals were randomly assigned to the different treatment groups. All animal experiments have been carried out with IACUC committee approval.

Antisense Phosphorothioate Oligodeoxynucleotides (AS-ODNs)

Antisense and control ODNs (20-mers) were customer synthesized by Geno Mechanix (Alachua, FL). AS-ODNs V515 was complementary to 5' UTR just up-stream of the translation start site (AUG codon) of VEGF mRNA: 5' - CTC ACC CGT CCA TGA GCC CG - 3'. AS-ODNs. B460 was complementary to the translation start site (AUG codon) of bFGF mRNA: 5' TCC CGG CTG CCA TGG TCC CT 3'. A scramble ODNs of V515: 5' – CAC CCT GCT CAC CGC ATG GC - 3' was used as ODNs controls. In the tumor up-take studies, Fluorescein isothioicyanate (FITC) was labelled at 5' end of VEGF scramble ODNs. All AS-ODNs were suspended in sterile and endotoxin free water at a concentration of 1 mM, aliquoted and stored at -20°C.

DOTAP:DOPE Liposome Preparation

Cationic liposomes were prepared using the method described by Tang (Tang and Hughes, 1999). Briefly, cationic lipid 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) was dissolved in chloroform and mixed with a helper lipid 1,2-dioleoyl-3-sn-phosphatidylethanolamine (DOPE) (Avanti Polar-Lipids, Alabaster, Al) at a molar ratio of 1:1. The mixture was evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The resulting lipid film was dried by nitrogen for an additional 10 min to evaporate any residual chloroform. The lipid film was re-suspended in sterile water to a final concentration of 1 mg/ml based on the weight of cationic lipid. The resultant mixtures were shaken in a water bath at 35°C for 30 min. The suspensions then were sonicated using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) for 1 min at room temperature to form homogenized liposomes. The particle-size distribution of liposomes was measured using a NICOMP 380 ZLS instrument (Santa Barbara, CA). The average particle diameter was 144.0 ± 77.0 nm. Liposomes were stored at 4°C and used within 3 months.

FACS Analysis of ODNs Tissue Distribution *In Vivo*

FITC labeled control ODNs were mixed with DOTAP:DOPE in 200 µl 5% dextrose and injected into Caki-1 xenograft bearing mice *via* the tail vein at a dose of 20 mg/kg. 24 hr later, the mice were killed by CO_2 asphyxiation, various tissues were collected, including spleen, liver, kidney, lung and tumor. The tissues were enzymatically dissociated into single cell suspension, using an enzyme cocktail of 0.02% DNase, 0.025% collagenase and 0.025% pronase (Allalunis-Turner and Siemann, 1986). All samples were then fixed in 1% p-formaldehyde for 24 hours and kept in dark. The

fluorescent intensities of the samples were analyzed by FACS on a Beckman Dickinson flow cytometer made available through the University of Florida Core Facility for Flow Cytometry.

Fluorescent Microscopy of ODNs Up-take *In Vivo*

FITC labeled control PS-ODNs were mixed with DOTAP:DOPE in 200 µl 5% dextrose and injected into Caki-1 xenograft bearing mice *via* the tail vein at a dose of 20 mg/kg. Three hours later, the mice were killed by CO_2 asphyxiation, lung, liver, spleen and kidney, as well as the tumors removed, quick frozen in liquid nitrogen and 20 µm sections were cut. Fluorescent field pictures of these sections were taken using a Zeiss Axioplan 2 Florescence Microscope (Zeiss, Thornwood, NY) within 3 days.

VEGF and bFGF Western Blots

VEGF AS-ODNs V515, bFGF AS-ODNs B460 or scramble control ODNs were injected *via* tail vein at a dose of 10 mg/kg. At various times after injection (24, 48 and 72 hr), the mice were killed, the tumors excised and quick frozen in liquid nitrogen. The tumors were then homogenized (Dounce tissue grinder, Wheaton, Millville, NJ) and the homogenates were lysed on ice for 30 min with 1 ml of hypotonic buffer (20 mM Tris-HCl, pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% Nonidet P-40, 2 mM Phenylmethanesulphonyl fluoride (PMSF), 200 U/ml Approtinin, 2 µg/ml leupetin) (Giannakakou et al., 1998) per 0.1 g tissue. Following a brief but vigorous vortex the samples were centrifuged at 14,000 rpm for 10 min at 4°C. A 30 µl aloquot of each sample was mixed with 10 µl 4x SDS-PAGE sample buffer (0.3 M Tris-HCl, pH 6.8, 45% glycerol, 20% β-mercaptoethanol, 9.2% SDS and 0.04 g/100ml bromophenol blue) and heated at 100°C for 10 min. 30 µl of each sample was then analyzed by SDS-PAGE

on a 12% separating gel and 3% stacking gel. Following transfer, the membrane was immunoblotted using a VEGF primary antibody (Sigma, Saint Louis, Missouri) 1:1000 or bFGF primary antibody (Upstate Biotechnology, Lake Placid, NY) 1:1000 diluted in antibody solution (3% dry milk, 25 mM Tris, pH 7.5, 0.5 M NaCl, 0.05% Tween20) overnight at 4°C. After washing, a secondary antibody labeled with horseradish peroxidase was applied and incubated at room temperature for 1 hr. Protein bands were visualized and densitometry was performed.

Tumor Growth Delay Assay

Once the Caki-1 xenografts reached a size of ~200 mm^3, animals were assigned randomly to various treatment groups. V515, B460 or control ODNs were administrated *via* the tail vein with DOTAP:DOPE liposomes at doses of 5 mg/kg or 10 mg/kg on day 1 and day 4. Tumors were measured using callipers and volumes were approximated by the formula, volume=$1/6\pi ab^2$, with a and b represent two perpendicular tumor diameters. The times for the tumors in the various treatment groups to grow from 200 mm^3 to 1000 mm^3 were recorded and compared (Wilcoxon rank sum test).

Results

The cationic liposome based delivery system was previously shown (Chapter 2) to efficiently deliver AS-ODNs into cultured Caki-1 cells. To evaluate the tissue distribution and tumor up-take of AS-ODNs, FITC labeled ODNs were mixed with cationic liposome DOTAP:DOPE in 5% dextrose and were injected *via* the tail vein at a dose of 20 mg/kg into Caki-1 xenograft bearing nude mice. 24 hr later, significant up-take of FITC-ODNs indicated by increased fluorescent intensity were observed in lung, liver, kidney, spleen and tumor, with liver having the highest up-take (Figure 5-1). Frozen sections prepared 3

hr later also showed that the FITC-ODNs were efficiently taken up in the Caki-1 tumor (Figure 5-2). The heterogeneous ODNs distribution likely reflects the inhomogeneous blood vessel network inside the tumor. Significant up-take of FITC-ODNs was also observed in normal tissues, such as lung, liver, kidney and spleen (Figure 5-3). Unlike tumor, normal tissue up-take appeared to be rather uniform. These findings suggested that the cationic liposome (DOTAP:DOPE) based delivery system can also serve as an efficient vehicle for systemic delivery of AS-ODNs *in vivo*.

To investigate whether the delivery of AS-ODNs was sufficient to suppress the target growth factor expression levels, tumor samples were collected at various times after systemic delivery of B460 and V515. Western blot analysis of these samples showed significant reductions in VEGF levels at 24, 48 and 72 hr post injection, with the maximum depression occurring at 48 hr after treatment (Figure 5-4). When B460 was administrated, significant and similar reductions in bFGF levels were observed at 48 and 72 hr (Figure 5-5). These findings clearly indicate that the delivery of VEGF and bFGF antisense to tumors can lead to significant reductions in VEGF and bFGF expression levels in Caki-1 xenografts.

To determine whether these reductions of pro-angiogenic growth factor production by AS-ODNs was sufficient to have anti-tumor efficacy *in vivo*, the effect of VEGF/bFGF AS-ODNs treatments on Caki-1 tumor growth was examined. Caki-1 xenografts-bearing mice were treated with two doses of VEGF (V515) or bFGF (B460) AS-ODNs (5 or 10 mg/kg) 1 and 4 days after the tumors reached a size of ~200 mm^3. The time for the tumors to grow from 200 mm^3 to 1000 mm^3 then was recorded (Figure 5-6). The results showed that the median time for the tumors to grow to 5 times the

starting size was significantly prolonged in the V515 or B460 treated groups. Administrating two doses of 5 mg/kg V515 caused a growth delay of ~4 days ($p<0.05$, Wilcoxon rank sum test) while treatment with two 10 mg/kg doses led to a tumor growth delay of ~6.5 days. Two doses of 5 mg/kg B460 caused a growth delay of ~5.5 days ($p<0.05$, Wilcoxon rank sum test) whereas two 10 mg/kg doses led to a tumor growth delay of ~10.5 days ($p<0.05$, Wilcoxon rank sum test). The higher dose treatments of both V515 and B460 therefore resulted in an approximately doubled the response of the tumors compared to the tumors of untreated or scramble ODNs treated mice to grow to the 5x starting size endpoint (Figure 5-6).

Discussion

The increased understanding of the biology of neoplasia and the identification, cloning, and sequencing of genes critical to tumor cell function permits the exploitation of this information to develop specific agents that may directly modulate the function of these genes or their protein products. Antisense oligonucleotides (AS-ODNs) are being investigated in this manner. However, ODNs are negatively charged large molecules that would result in poor cellular uptake and intracellular distribution. A reasonable solution to this problem is the use of cationic liposome based delivery methods, which have become the standard for *in vitro* AS-ODNs experiments (Capaccioli et al., 1993; Lappalainen et al., 1994) (see also Chapter 2). However for *in vivo* delivery of AS-ODNs, methods are still being standardized. To date, *in vivo* ODNs solutions delivery approaches have relied on saline (Agrawal et al., 1995; Dean and McKay, 1994; Nesterova and Cho-Chung, 1995; Akhtar and Agrawal, 1997; Yazaki et al., 1996; Whitesell et al., 1991). Despite the apparent success of some ongoing trials using such

approaches, it is generally believed that improved delivery to specific tissues will prove advantageous and lead to higher success rates. Cationic liposomes which have been used successfully *in vitro* are possible candidates for *in vivo* delivery of AS-ODNs. Evidence for their utility already exists (Zhang et al., 2000; Chen et al., 2000; Yang et al., 1998). This approach may be even more preferable for the treatment of solid tumors. Small molecule agents have a large volume of distribution after i.v. administration (Chabner B.A. and Longo D.L., 1996). Unfortunately, the result of this is often a narrow therapeutic index due to a high level of toxicity in healthy tissues. By encapsulating the AS-ODNs with liposomes, the complexes become macromolecular. The volume of distribution therefore is significantly reduced and may result in a decrease of nonspecific toxicities. There also is believed to be an increase in the amount of drug that can be effectively delivered to the tumor (Lasic and Papahadjopoulos, 1995; Gabizon and Martin, 1997). Under optimal conditions, the drug is carried within liposomal aqueous space while in the circulation but leaks out at a sufficient rate to become bio-available on arrival at the tumor. The liposome protects the drug from metabolism and inactivation in the plasma, and due to the size limitations in the transport of large molecules across healthy endothelium, the drug accumulates to a reduced extent in healthy tissues (Mayer et al., 1989; Vaage et al., 1994). The junctions in the vascular endothelium of healthy tissues vary depending on the types of tissue (Seymour, 1992). In most tissues, including connective tissue and tissues of muscle, heart, brain, and lung, intercellular tight junctions result in openings <2 nm. Though these openings can approach 6 nm in post-capillary venules, they are still considerably smaller than the size of liposomes (usually 65-150 nm) (Seymour, 1992; Lum and Malik, 1994). For organs or tissues with discontinuous

endothelium, such as fenestrated endothelium of the kidney glomerulus or the sinusoidal endothelium of the liver and spleen, they can have junctions ranging from 40-60 nm for the former and up to 150 nm for the latter (Seymour, 1992). Still, most liposome formulations are larger than the threshold required for glomerular filtration. However, liposomes are able to enter tumors effectively due to a discontinuous tumor microvasculature, where pore sizes vary between 100 to 780 nm in size (Yuan et al., 1995; Hobbs et al., 1998). The selective accumulation in tumors is thus made possible by the impervious nature of the endothelium of most healthy tissues.

Bearing this belief in mind, experiments were carried out to evaluate delivery efficiency of FITC-ODNs by cationic liposome (DOTAP:DOPE) in Caki-1 xenograft tumor bearing mice. Fluorescent intensity studies of different tissues showed that AS-ODNs were delivered into multiple organs, and most importantly, into the xenograft tumor as well (Figure 5-1). The histological section of the tissues further confirmed that efficient deliveries were achieved in liver, kidney, lung and spleen (Figure 5-3). Significant up-take of AS-ODNs, though heterogeneous, was also observed in Caki-1 tumors. This is believed to be due to the heterogeneous distribution of the tumor vascular network (Figure 5-2). Nonetheless, cationic liposomes cleared proved to be an efficient delivery vehicle for AS-ODNs *in vivo*.

Western blot studies of the tumor homogenates confirmed that using such delivery system, sufficient AS-ODNs were taken-up by the tumors to suppress VEGF and bFGF protein expression (Figure 5-4 and 5-5). The fact that maximum suppression of VEGF protein levels was observed earlier than that of bFGF levels is believed due to the short half life of VEGF *in vivo* (Ferrara, 1999). More importantly, this suppression of

pro-angiogenic growth factors was focused to result in significant tumor growth delays in the Caki-1 tumor model (Figure 5-6). These results provide the first experimental evidence that the systemic treatment of mice bearing macroscopic tumors with bFGF AS-ODNs can have substantial antitumor efficacy, and that VEGF AS-ODNs are effective in treating a VEGF independent tumor model. In both cases significant tumor growth delays could be achieved without overt toxicity and with doses well below the LD10 dose (Figure 5-6).

The results of this study indicate a key role for the VEGF and bFGF signalling pathway in RCC angiogenesis. It is clear that interfering with VEGF and bFGF signaling can disrupt the tumor induced angiogenesis which in turn leads to tumor growth inhibition. Specially, AS-ODNs treatments provided a viable measure to modulate tumor angiogenesis and resulted in significant growth delays in this VEGF independent RCC tumor model. Taken together, these findings suggest that AS-ODNs targeted to VEGF and bFGF may have utility in the management of RCC. It may also possibly provide therapeutic benefit when used in conjunction with other anti-cancer therapies.

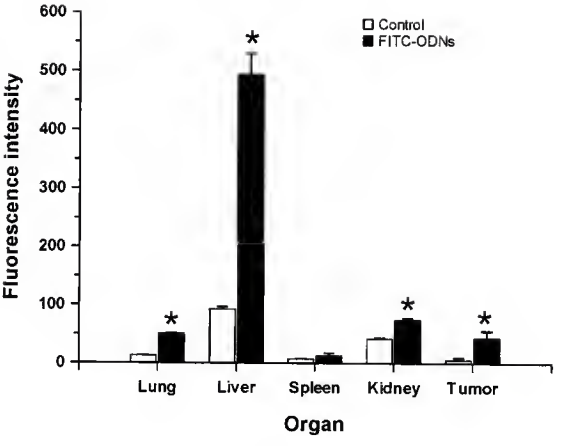


Figure 5-1. Fluorescence intensity of different organs before and after i.v. injection of 20 mg/kg FITC labeled ODNs. Each bar represents the mean ± S.E. of samples from 6 animals. Stars indicate statistical significance compared to untreated control animals ($p<0.05$, student's *t*-test)

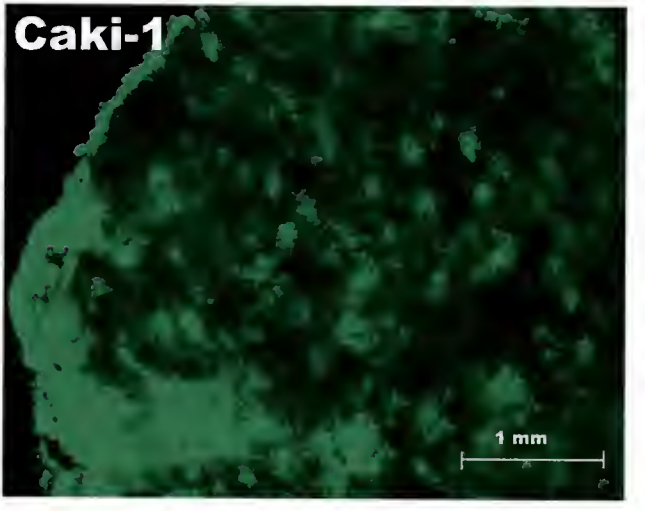


Figure 5-2. Representative picture of uptake and distribution of FITC-labeled ODNs in Caki-1 xenografts determined 3 hr after i.v. injection of a 20 mg/kg dose.

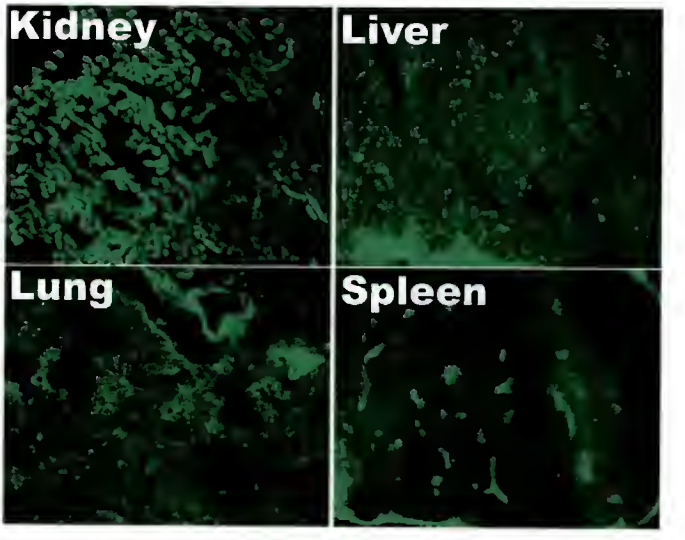


Figure 5-3. Representative pictures of uptake and distribution of FITC-labeled ODNs in liver, lung, kidney and spleen determined 3 hr after i.v. injection of a 20 mg/kg dose.

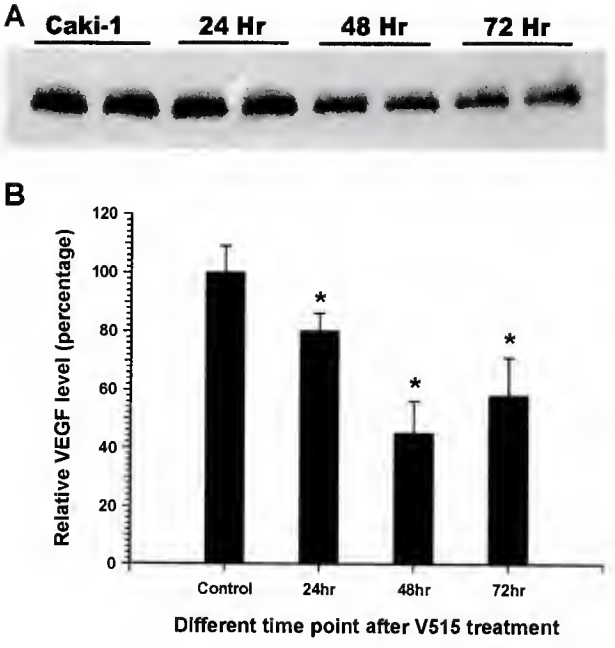


Figure 5-4. VEGF protein levels in Caki-1 xenografts at various times after treatment with 10 mg/kg VEGF AS-ODNs. A) representative VEGF western blot results; B) relative VEGF levels in Caki-1 tumors. Each bar represents the mean ± S.E. of six tumors. Stars indicate statistical significance compared to untreated control tumors ($p<0.05$, student's *t*-test).

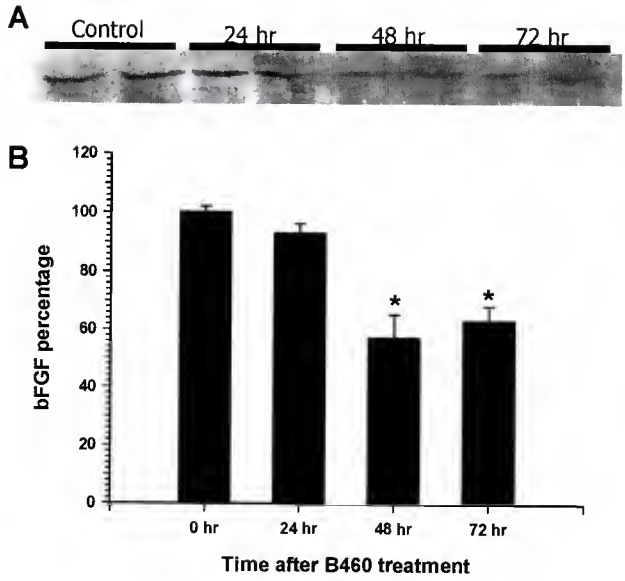


Figure 5-5. Protein levels of bFGF in Caki-1 xenografts at differet times after treatment with 10 mg/kg bFGF AS-ODNs. A) representative bFGF western blot results; B) relative bFGF levels of Caki-1 tumors. Each bar represents the mean ± S.E. of six tumors. Stars indicate statistical significance compared to untreated control tumors ($p<0.05$, student's *t*-test).

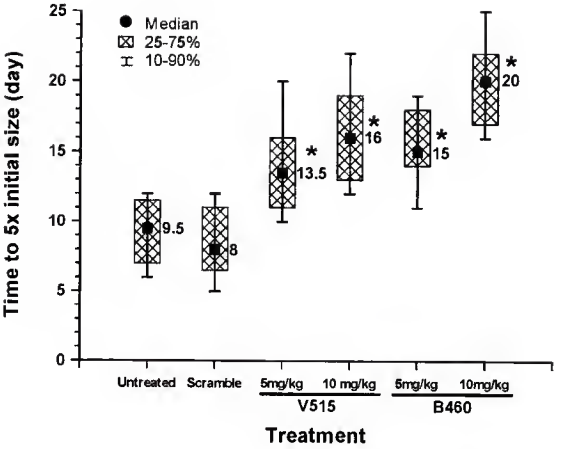


Figure 5-6. Tumor response of Caki-1 tumors treated systemically with VEGF or bFGF AS-ODNs or controls. Each group contains 10 animals. Stars indicate statistical significance compared to untreated control ($p<0.05$, Wilcoxon rank sum test).

CHAPTER 6
COMBINATION STUDIES

Introduction

It has been established that solid tumors cannot grow beyond microscopic size without inducting a new vascular network. Development of novel anti-tumor agents that exploit this observation and suppress angiogenesis is an active area of cancer research. Angiogenesis is a complex process with multiple, sequential and interdependent steps, this complexity also creates many potential targets for therapeutic intervention. Many agents that are antiangiogenic have been identified and characterized (Folkman, 1985; Moses and Langer, 1991; Schweigerer, 1995; Scott and Harris, 1994; Fan et al., 1995; Kerbel, 2000; Sauer et al., 2002). Each of these affect at least one of the several stages involved in new vessel formation, i.e. basement membrane degradation, endothelial cell migration, endothelial cell proliferation, and tube formation.

Although strategies aimed at compromising tumor angiogenesis have demonstrated significant ant-tumor effect in preclinical investigations, achieving tumor cures in patients with anti-angiogenic agents is likely to be extremely difficult (Folkman, 1985; Moses and Langer, 1991; Schweigerer, 1995; Scott and Harris, 1994; Fan et al., 1995). The complexity of pathways available for angiogenesis probably means that disrupting only a single aspect probably will not be enough. It would seem likely that targeting one factor, for example VEGF, would result in the eventual selection of tumor cell variants which could induce angiogenesis through production of other growth factors (Yoshiji et al., 1997). Indeed there is evidence that as tumors progress, the number of

different pro-angiogenic growth factors produced by the tumor increases (Relf et al., 1997; Eggert et al., 2000). Moreover, in addition to angiogenesis dependent tumor growth, angiogenesis independent tumor growth can occur in human tumors (Maniotis et al., 1999). One study of non-small-cell lung tumors found up to 16% of the tumors appeared to grow along pre-existing blood vessels. The tumors did not exhibit signs of angiogenesis but the microvessel density appeared similar to that seen in angiogenic tumors (Pezzella et al., 1997). Other studies also revealed "co-opt" of vessels during early growth by tumors where evidence of angiogenesis was absent (Holash et al., 1999; Burke and DeNardo, 2001). Therefore strategies aimed at multiple steps and different growth factors involved in angiogenesis may offer enhanced efficacy and long lasting effects.

Moreover, the over-arching goal of anti-angiogenic strategies is to interfere with pro-angiogenic balance between tumor, stromal and endothelial cells in order to stabilize the growth of the tumor by preventing further development of a functional vessel network. Therefore, theoretically the optimal treatment outcome of anti-angiogenic strategies would be tumor growth arrest rather than tumor killing. In order to eliminate the existing pockets of tumor cells, cytotoxic agents need to be used in conjunction with such therapies. These considerations, along with the extensive clinical use of conventional anti-cancer therapy, make the thorough investigation of strategies combining different anti-angiogenic approaches as well as combinations with conventional treatment modality imperative.

In the current studies, the anti-tumor efficacy of VEGF/bFGF AS-ODNs treatment in combinations with other anti-angiogenic strategies, as well as a conventional cytotoxic therapy, radiotherapy, were evaluated.

Materials and Methods

Caki-1 Xenografts

Female nude mice (NCR, nu/nu), age 6 - 8 weeks were maintained under specific-pathogen-free conditions (University of Florida Health Science Center) with food and water supplied *ad libitum*. Initial Caki-1 xenografts were established by inoculating 5×10^6 Caki-1 tumor cells subcutaneously into the flanks of nude mice. Because of the rather long time for the xenografts to appear and grow, and the relatively low take rate, subsequent passages of Caki-1 tumors were achieved by implanting tumor pieces subcutaneously in the flanks of recipient animals. To initiate tumors in the leg muscle, animals were inoculated intramuscularly in the left hind leg with 2×10^6 tumor cells suspended in 20 µl 0.9% saline chloride. When the tumors reached a size ~200 mm^3, animals were randomly assigned to the different treatment groups. All animal experiments have been carried out with IACUC committee approval.

Antisense Phosphorothioate Oligodeoxynucleotides (AS-ODNs)

Antisense and control ODNs (20-mers) were customer synthesized by Geno Mechanix (Alachua, FL). AS-ODNs V515 was complementary to 5' UTR just up-stream of the translation start site (AUG codon) of VEGF mRNA: 5' - CTC ACC CGT CCA TGA GCC CG - 3'. AS-ODNs B460 was complementary to the translation start site (AUG codon) of bFGF mRNA: 5' TCC CGG CTG CCA TGG TCC CT 3'. A scramble sequence of V515: 5' – CAC CCT GCT CAC CGC ATG GC - 3' was used as control ODNs. All AS-ODNs were suspended in sterile and endotoxin free water at a concentration of 1 mM, aliquoted and stored at -20°C.

DOTAP:DOPE Liposome Preparation

Cationic liposomes were prepared using the method described by Tang (Tang and Hughes, 1999). Briefly, cationic lipid 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) was dissolved in chloroform and mixed with a helper lipid 1,2-dioleoyl-3-sn-phosphatidylethanolamine (DOPE) (Avanti Polar-Lipids, Alabaster, Al) at a molar ratio of 1:1. The mixture was evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The resulting lipid film was dried by nitrogen for an additional 10 min to evaporate any residual chloroform. The lipid film was re-suspended in sterile water to a final concentration of 1 mg/ml based on the weight of cationic lipid. The resultant mixtures were shaken in a water bath at 35°C for 30 min. The suspensions then were sonicated using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) for 1 min at room temperature to form homogenized liposomes. The particle-size distribution of liposomes was measured using a NICOMP 380 ZLS instrument (Santa Barbara, CA). The average particle diameter was 144.0 ± 77.0 nm. Liposomes were stored at 4°C and used within 3 months.

PD0203359-0002 Drug Preparation

PD0203359-0002 (Pfizer, Warner Lambert, Ann Harbor, MI), 1-tert-butyl-3-[6-(2-chloro-3,5-dimethoxy-phenyl)-2-(4-diethylamino-butylamino)-pyrido [2,3-d] pyrimidin-7-yl]-urea hydrochloride, was prepared in 0.05 M sodium lactate buffer (pH 4.0) at a concentration of 10 mg/ml. The drug solution was stored at 4°C and used within a week.

Local Tumor Irradiation

Irradiations were performed using a 6MV Clinac 600c linear accelerator (Varian Oncology Systems, Palo Alto, CA), operating at a dose rate of 400 cGy/min, on unanesthetized mice confined in plastic jigs. The animals' tumor-bearing legs extended through openings in the sides of the jigs allowing the tumors to be irradiated locally.

Tumor Growth Delay Assay

Tumor response to treatment was determined using a growth delay assay. Tumors initiated in the hind legs were measured every second day by passing the tumor-bearing leg through a series of increasing diameter holes in a plastic plate. The smallest diameter hole the tumor-bearing leg could pass through was recorded and converted to a tumor volume using the formula: tumor volume = $1/6\pi d^3$-100, where d = hole diameter and 100 represent a volume correction factor determined for a mouse leg without a tumor. Tumors established subcutaneously in the flank were measured using callipers and volumes were approximated by the formula, tumor volume=$1/6\pi ab^2$, with a and b represent two perpendicular tumor diameters. The times for the tumors in the various treatment groups to grow to 200 mm^3 and from 200 mm^3 to 1000 mm^3 were recorded and compared (Wilcoxon rank sum test).

Results

Given the encouraging positive results of systemic administration of VEGF/bFGF AS-ODNs in the human tumor xenograft model of RCC (chapter 5), experiments were first carried out to examine the efficacy of combining VEGF and bFGF AS-ODNs treatments. However, combining 2 doses of 10 mg/kg V515 and B460 did not yield a significantly greater tumor response than did B460 treatment alone (Figure 6-1). Since

B460 AS-ODNs treatment affects Caki-1 cell proliferation and apoptosis (chapter 4), it is apparent that this treatment has direct anti-tumor effects in addition to the anti-angiogenic effects. It was postulated that multiple exposures might be required to demonstrate the effect. This hypothesis was confirmed in the experiment with more treatment doses. When 4 doses of V515 and B460 were administrated, a moderate but significant enhancement in tumor growth delay was observed (Figure 6-2). The results showed that combination of two different AS-ODNs (V515 and B460) could provide a moderate increase in the tumor response. However this approach may lead to increased toxicity and side effects due to the common delivery vehicle utilized and common metabolic pathway involved. It would appear more reasonable and appealing to combine AS-ODNs treatments with other anti-angiogenic therapies or conventional anti-cancer treatment.

VEGF and bFGF signaling play central roles in tumor angiogenesis. Besides directly targeting VEGF/bFGF proteins through the use of AS-ODNs, blocking their specific receptor tyrosine kinase activities offers another way to disrupt the signaling pathway. The experimental compound PD0203359-0002 (Figure 6-3) was found to specifically inhibit the VEGF and bFGF receptor tyrosine kinase activities *in vitro* and *in vivo*. Oral administration of this compound at doses of 20 mg/kg and 40 mg/kg to Caki-1 xenograft bearing mice for 14 days resulted in dose dependent tumor growth delay (Figure 6-4). However, considerable side effects and toxicity were observed in the higher dose treatment group, as indicated by significant weight loss (up to 30%), which limited its potential use. However, when the lower dose treatment of PD0203359-0002 (20 mg/kg) was administrated in conjunction with VEGF or bFGF AS-ONDs, significantly

enhanced tumor responses were observed (Figure 6-5). These effects were achieved at non-toxic doses of either treatment.

Lastly, the VEGF and bFGF AS-ODNs treatment in combination of with radiation was evaluated. VEGF and bFGF AS-ODNs were given as 2 doses after locally irradiating the tumors with a single dose of 10 Gy. The result showed that the combination treatment led to significantly prolonged tumor responses (Figure 6-6).

Discussion

It has been suggested that combining anti-angiogenic agents that work *via* different mechanisms may augment anti-tumor efficacy. For example, a VEGF receptor antibodies might be administrated with a conventional chemotherapeutic agents that are dosed in an "anti-angiogenic" schedule to enhance the tumor response (Browder et al., 2000; Hanahan et al., 2000; Klement et al., 2000). It is not surprising, given the complexity of tumor angiogenesis and redundancy of pro-angiogenic growth factors made by tumor cells, that multiple steps or targets of the angiogenic process need to be controlled in order to achieve long-term tumor control. In the RCC tumor, multiple pro-angiogenic growth factors are found to be over-expressed. These include VEGF, bFGF, PDGF, etc (Wechsel et al., 2000; Takahashi et al., 1994; Mydlo et al., 1988; Relf et al., 1997). Therefore, exploring therapies in this model seems very logical.

Combining VEGF and bFGF AS-ODNs was found effective only when multiple doses were used over a prolonged treatment course (Figure 6-1 and Figure 6-2). Because VEGF and bFGF are considered to be the most important pro-angiogenic growth factor in tumors, the combination studies were somewhat disappointing. Other studies have shown that VEGF and bFGF possess synergistic effects in the induction of angiogenesis (Goto et

al., 1993; Seghezzi et al., 1998; Mandriota and Pepper, 1997; Yoshiji et al., 2002). Suppression of VEGF by neutralizing antibody or AS-ODNs also resulted in inhibition of bFGF induced angiogenesis (Seghezzi et al., 1998; Majima et al., 2000). These findings suggest possible interactions between these two growth factors or involvement of the same signal transduction pathway. Moreover, since both factors were targeted using the AS-ODNs, it is possible that the delivery and up-take of AS-ODNs into the tumors was saturated. Besides, bFGF also plays an important role in the RCC cell growth. Blocking bFGF expression would lead to cell cycle inhibition of Caki-1 cells and also induce apoptotic cell death (chapter 3). Therefore combining the AS-ODNs targeted to both VEGF and bFGF may only yield a moderate gain, especially compared to bFGF AS-ODNs treatment alone. Agents that target different steps in angiogenic process or work under different mechanisms should provide increased efficacy without possible increases in toxicity.

One such approach is to combine AS-ODNs targeted to VEGF/bFGF with a strategy to specially block VEGF/bFGF receptors. This was achieved by combining V515/B460 AS-ODNs treatment with PD0203359-0002 administration. This approach targeted two different cell populations and two different aspects of the angiogenic process by inhibiting the growth factors produced by tumor cells and the receptor function of the endothelial cells. Results showed additive enhancement of tumor responses with such combination (Figure 6-5). Importantly, the enhanced responses were achieved at the low, non-toxic dose of PD0203359-0002. These findings support the principle that targeting multiple angiogenic steps using different therapeutic approaches may offer greater anti-tumor effects without increasing the risks of toxicity.

Since anti-angiogenic therapies should not result in tumor kill on their own, the resumption of tumor growth when therapy is discontinued represents a significant potential problem. A logical solution to this problem is the combination of such treatments with conventional cytotoxic agents such as chemotherapy or radiotherapy. Evidence already exists that combining anti-angiogenic agents with chemotherapeutic agents can improve the treatment efficacy (Lee et al., 1987; Bertolini et al., 2000; Hanahan, 1998; Klement et al., 2002; Inoue et al., 2000). However, RCC is usually resistant to chemotherapy. A review of 83 clinical trials with 4,542 patients showed a low overall response to treatment (6.8%; 4.7% partial response rate and 1.3% complete response rate) (Vogelzang and Stadler, 1998). It therefore seems more reasonable to combine the current anti-angiogenic therapy with cytotoxic therapy, like radiation in the tumor model of RCC. Although, RCC is typically considered a radio-resistant tumor as well, it has been proposed that radiation therapy may provide some benefits to patients. Pre-operative radiation therapy may provide benefits such as: 1) reduction of the risk of tumor dissemination at the time of surgery; 2) reduction the size of primary tumor; 3) increased resectability; 4) reduction of tumor vasculature (Cox et al., 1970; Kortmann et al., 1999). Moreover, postoperative radiation therapy has the theoretical benefit of providing local tumor control in patients with positive surgical margins, incompletely resected tumor or lymph node involvement (Makarewicz et al., 1998; Stein et al., 1992; Riches, 1966). Besides, tumor progression during the course of treatment is a major reason for radiotherapy failures. And the ability of tumors to progress is dependent on the formation of new blood vessels. Consequently, the application of anti-angiogenic strategies that disrupt the pro-angiogenic balance between neoplastic, stromal and

endothelial cell may result in growth stabilization of the tumor by preventing further development of a functional vessel network.

This concept was confirmed in the present studies. When VEGF and bFGF AS-ODNs treatment were combined with a single local radiation dose of 10 Gy, augmented tumor responses were observed (Figure 6-6). The effect appears to be additive. The mechanisms underlying the enhancement of the radiation response by the anti-angiogenic therapies likely include an increase in tumor oxygenation (Lee et al., 2000; Hess et al., 2001; Teicher et al., 1995), a decrease in vascular density (Lee et al., 2000), and possibly the radio-sensitization of endothelial cells (Gorski et al., 1999; Mauceri et al., 1998). While there has been some concern that the inhibition of tumor angiogenesis might increase the fraction of hypoxic tumor cells and thereby induce radiation resistance, this concern is not relevant in the present studies, since the anti-angiogenic agents (V515 and B460) were administrated after the radiation exposure. Also, the concern of induction of hypoxia is not supported by evidence of improved tumor oxygenation after treatment (Lee et al., 2000; Hess et al., 2001; Teicher et al., 1995), as well as studies showing that blocking the VEGF signaling pathway improves radiotherapy response under both fully oxygenated and hypoxic conditions (Lee et al., 2000). Still, it is an important issue that is strongly dependent on the agent under investigation and one that will warrant rigorous scrutiny.

These preliminary results clearly indicate that combining different anti-angiogenic agents or combining anti-angiogenic with conventional anti-cancer therapies may provide significant improvements in treatment outcomes and hence should be thoroughly explored.

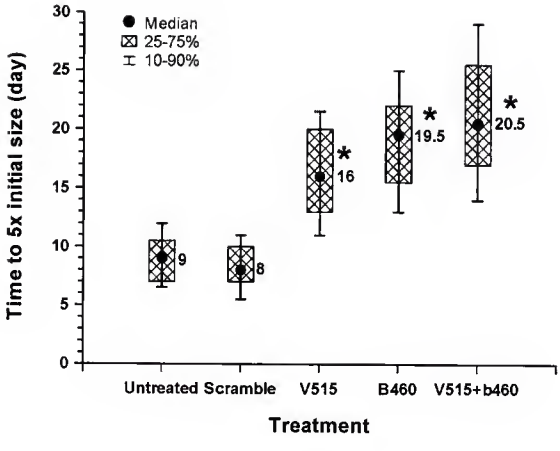


Figure 6-1. Tumor responses of Caki-1 xenografts after treatment with VEGF and bFGF AS-ODNs. Two doses of V515 and B460 (10 mg/kg each) were given on day 1 and day 4. Each group contained 10 animals. Stars indicate statistical significance compared to untreated control animals ($p<0.05$, Wilcoxon rank sum test).

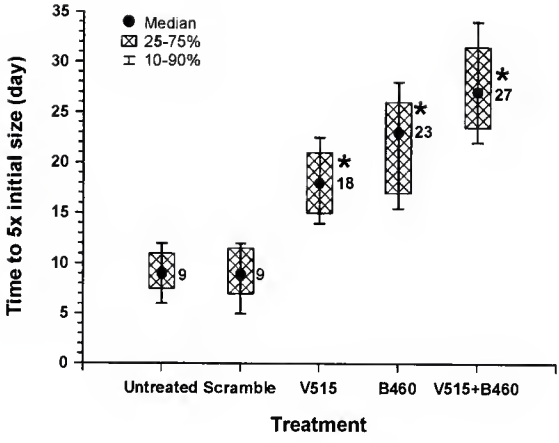
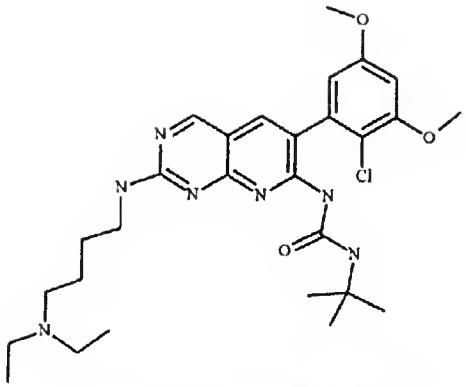


Figure 6-2. Tumor responses of Caki-1 xenografts after treatment with VEGF and bFGF AS-ODNs. V515 and B460 were given as 4 doses of 10 mg/kg on day 1, 4, 7 and 10. Each group contained 10 animals. Stars indicate statistical significance compared to untreated control animals ($p<0.05$, Wilcoxon rank sum test). V515+B460 treated group is statistically significant compared to V515 or B460 treated groups ($p<0.05$, Wilcoxon rank sum test).



PD 0203359-0002

Figure 6-3. Chemical structure of VEGF/bFGF receptor tyrosine kinase inhibitor PD0203359-0002.

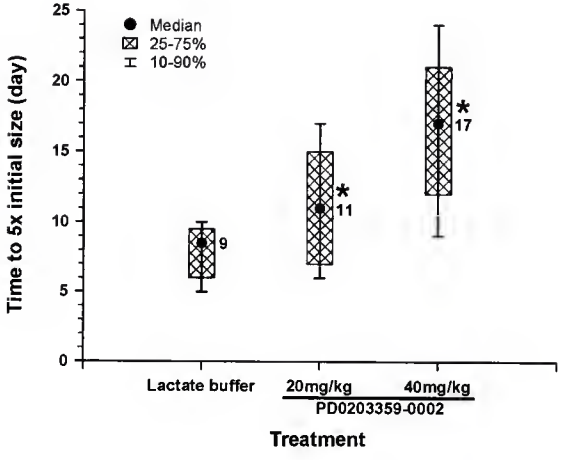


Figure 6-4. Tumor responses of Caki-1 xenografts after treatment with PD0203359-0002. PD0203359-002 was given daily at the doses indicated for 14 days. Each group contained 10 animals. Stars indicate statistical significance compared to vehicle treated control animals (lactate buffer) ($p<0.05$, Wilcoxon rank sum test).

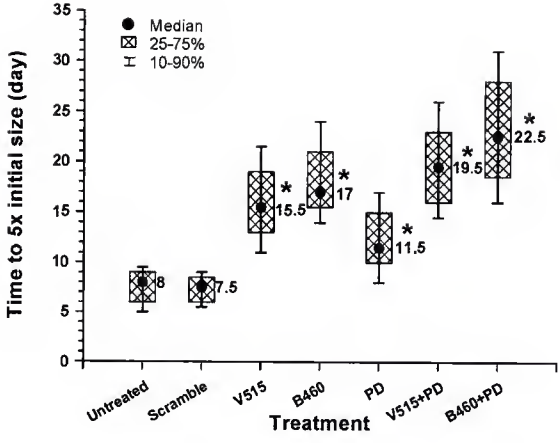


Figure 6-5. Tumor responses of Caki-1 xenografts after treatment with VEGF/bFGF AS-ODNs and PD0203359-0002. V515 and B460 were given at 2 doses of 10 mg/kg on day 1 and day 4. PD0203359-0002 was given daily at a dose of 20 mg/kg for 14 days. Each group contained 10 animals. Stars indicate statistical significance compared to untreated control animals ($p<0.05$, Wilcoxon rank sum test). V515+PD treated group is significantly different compared to V515 or PD treated groups; B460+PD treated group is significantly different compared to B560 or PD treated groups ($p<0.05$, Wilcoxon rank sum test).

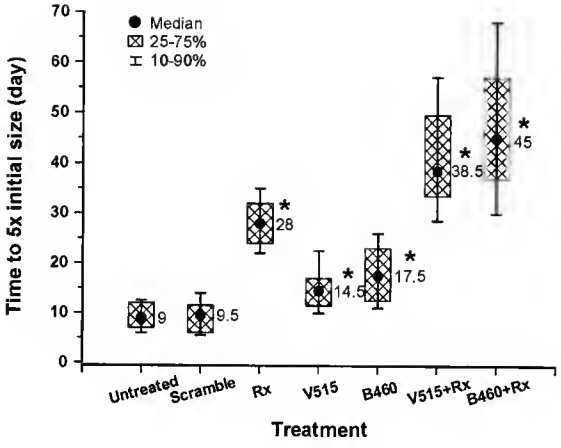


Figure 6-6. Tumor responses of Caki-1 xenografts after treatment with VEGF/bFGF AS-ODNs and radiation. V515 and B460 were given as 2 doses of 10 mg/kg on day 1 and day 4. Local radiation of 10 Gy was given 1 hr before the first dose of AS-ODNs. Each group contained 10 animals. Stars indicate statistical significance compared to untreated control animals ($p<0.05$, Wilcoxon rank sum test). V515+Rx and B460+Rx treated groups are significantly different compared to Rx, V515 or B460 treated groups ($p<0.05$, Wilcoxon rank sum test).

CHAPTER 7
SUMMARY AND PERSPECTIVE

Angiogenesis is a critical pathogenic step in the growth and metastatic dissemination of solid tumors (Folkman, 2001). It is a complex process involving multiple steps and pathways highly dependent on the local balance between positive and negative regulatory factors. It is now well established that the majority of tumor blood vessels are newly formed as a result of angiogenesis triggered by the release of stimulators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Strategies for therapeutic suppression of angiogenesis have focused extensively on the various aspects of the process of angiogenesis. Many agents that are anti-angiogenic have been identified and characterized; each of these affect at least one of the several stages involved in new vessel formation, i.e. basement membrane degradation, endothelial cell migration, endothelial cell proliferation and tube formation.

Pro-angiogenic growth factors VEGF and bFGF are primary targets because of they play central roles in tumor angiogenesis. Strategies targeting VEGF/bFGF signaling vary from using antibodies against VEGF/bFGF proteins or their receptors, over-expressing soluble receptors, and interference with VEGF/bFGF binding to their receptors to inhibit their receptor tyrosine kinase activities. Antisense technology offers another way to specifically target these growth factors producted by the tumor cells. The efficacy of antisense oligodeoxynucleotides targeted against VEGF/bFGF treatments on the angiogenesis and growth of tumors was evaluated in this dessertation.

Renal cell carcinoma (RCC) is the most common malignancy of the kidney. Because of lacking specific symptoms for early detection and unfavorable treatment outcomes to conventional anti-cancer therapies in the advanced disease, it remains a challenge in modern oncology. A characteristic feature of RCC is evidence of abundant angiogenesis and abnormal blood vessel development. VEGF and bFGF are known contributors in the regulation of RCC initiated angiogenesis. Their expression levels also associated with the progression and prognosis of RCC. In the present studies we evaluated the effects of blocking VEGF and bFGF production by AS-ODNs on the growth and angiogenic activity in a pre-clinical model of RCC (Caki-1).

In order to achieve therapeutic effect, sufficient cellular uptake of AS-ODNs is required. A cationic liposome (DOTAP:DOPE) based delivery system was developed to enhance the delivery efficiency. By optimizing the liposome composition and liposome to ODNs charge ratio, efficient delivery of ODNs both *in vitro* and *in vivo* was achieved with a safe non-toxic dose.

AS-ODNs sequences against all isoforms of VEGF and bFGF were designed and effective sequences were identified (VEGF AS-ODNs,V515 and bFGF AS-ODNs, B460). *In vitro* studies showed that treating Caki-1 cells with antisense AS-ODNs directed against VEGF or bFGF mRNA led to a reduction in the levels of VEGF/bFGF expression. This suppression of these pro-angiogenic growth factors was sufficient to impair the proliferation and migration of co-cultured endothelial cells. In addition, bFGF AS-ODNs treatment also exerted a direct effect on Caki-1 cell growth. The observed effects were antisense sequence specific, dose dependent, and could be achieved at a low, non-toxic concentration of AS-ODNs. When VEGF/bFGF AS-ODNs treated Caki-1 cells

were injected into nude mice and evaluated for their angiogenic potential in an intradermal angiogenesis assay, the number of vessels initiated were approximately half that initiated by untreated Caki-1 cells. These results demonstrated that AS-ODNs treatment could be used to modulate the expression of pro-angiogenic growth factors, like VEGF and bFGF *in vivo*. It therefore was concluded that the suppression of VEGF or bFGF due to AS-ODNs treatment was sufficient to impair the tumor angiogenic process both *in vitro* and *in vivo*.

To evaluate the anti-tumor effects of AS-ODNs treatment, VEGF/bFGF AS-ODNs, V515 and B460 were administrated systemically by tail vein to nude mice bearing macroscopic Caki-1 xenografts. The results showed that two doses of VEGF or bFGF AS-ODNs given 1 and 4 days after the tumors reached a size of ~200 mm^3 approximately doubled the time required for tumors to grow to 5 times the size at the beginning of treatment. These findings indicate that a significant anti-tumor effect could be achieved with AS-ODNs targeted to pro-angiogenic growth factors.

Given the complexity of tumor angiogenesis, target one molecule or step in the process may ultimately not be sufficient to impair tumor growth. Increased efficacy without increased toxicity can be achieved when combining anti-angiogenic approaches against different aspects of angiogenesis or combining anti-angiogenic strategies with conventional anti-cancer therapies. When multiple doses of VEGF and bFGF AS-ODNs were combined, enhanced tumor growth delay was observed, however, the overall gain was moderate. This is believed to be due to the fact that both agents were delivered by and acted through the same mechanism, and this could saturate the effect that could be achieved. In contrast the combination of VEGF and bFGF AS-ODNs with VEGF/bFGF

receptor tyrosine kinase inhibitor (PD0203359-0002) showed significantly longer tumor growth delay without toxicity. Moreover, when VEGF/bFGF AS-ODNs treatment was combined with the conventional anti-cancer therapy, radiotherapy, an a additive response was observed. The findings indicate that using AS-ODNs as an anti-angiogenic strategy not only has utility as an effective anti-tumor treatment on its own, but also provides significant benefit in combination with other new or conventional anti-cancer therapies.

Given the encouraging results, there are still areas of interest that could be pursued in future preclinical studies. For example, combination of VEGF/bFGF AS-ODNs with other anti-angiogenic agents, such as endogenous angiogenic inhibitors like endostatin, angiogstatin. The combination of VEGF/bFGF AS-ODNs was evaluated with single dose radiation therapy. Future studies might focus on combination with fractionated radiation therapy, which is more relevant to the clinical setting. Further studies also need to carefully evaluate the impact of anti-angiogenic therapies on tumor oxygenation, and whether or how they could affect the effects of radiotherapy. Another important question is, what will be the best schedule for administrating of radiotherapy with anti-angiogenic therapies. Also, even though RCC is a chemo-resistant tumor, the possible benefits of combining VEGF/bFGF AS-ODNs treatment with chemotherapy in sensitive or responsive tumor models needs to be examined. Finally, the assessment of the effects of VEGF/bFGF AS-ODNs treatment on tumor metastasis will be worthwhile and ought to be tested in future preclinical investigations.

The research described in this dissertation has been presented at 6 national and international meetings. Portions of the studies have been published in *the British Journal of Cancer* (Shi and Siemann, 2002) and *Seminars in Radiation Oncology* (Siemann and

Shi, 2002). Another manuscript based on the studies of this work has been submitted for publication to *Anticancer Research*.

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BIOGRAPHICAL SKETCH

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